Enhanced Expression of Asparagine Synthetase under Glucose-Deprived Conditions Protects Pancreatic Cancer Cells from Apoptosis Induced by Glucose Deprivation and Cisplatin

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Abstract

Although hypovasculature is an outstanding characteristic of pancreatic cancers, the tumor cells survive and proliferate under severe hypoxic, glucose-deprived conditions caused by low blood supply. It is well known that the hypoxia-inducible factor-1 pathway is essential for the survival of pancreatic cancer cells under hypoxic conditions. To discover how pancreatic cancer cells adapt to glucose deprivation as well as hypoxia, we sought glucose deprivation–inducible genes by means of a DNA microarray system. We identified 63 genes whose expression was enhanced under glucose-deprived conditions at >2-fold higher levels than under normal glucose conditions. Among these genes, asparagine synthetase (ASNS) was studied in detail. Although it is known to be associated with drug resistance in leukemia and oncogenesis triggered by mutated p53, its function is yet to be determined. In this study, we found that glucose deprivation induced the overexpression of ASNS through an AMP-activated protein kinase–independent and activating transcription factor-4–dependent manner and that ASNS protects pancreatic cancer cells from apoptosis induced by glucose deprivation itself. ASNS overexpression also induced resistance to apoptosis triggered by cisplatin [*cis*-diammine-dichloroplatinum (CDDP)] and carboplatin, but not by 5-fluorouracil, paclitaxel, etoposide, or gemcitabine. We show that glucose deprivation induces the activation of c-jun NH_2-terminal kinase (JNK)/stress-activated protein kinase (SAPK) in a mock transfectant but not in an ASNS transfectant. Consequently, an inhibitor of JNK/SAPK decreased the sensitivity of pancreatic cancer cells to apoptosis by glucose deprivation and CDDP. These results strongly suggest that ASNS is induced by glucose deprivation and may play a pivotal role in the survival of pancreatic cancer cells under glucose-deprived conditions. [Cancer Res 2007;67(7):3345–55]

Introduction

As solid tumors outgrow the existing vasculature, they are continuously exposed to a microenvironment in which the supply of oxygen is limited. Adaptation to such a microenvironment through angiogenesis and increased glycolysis, two universal characteristics of solid tumors, is regulated by hypoxia-inducible factor-1 (HIF-1) and is critical to the sustenance of neoplastic proliferation (1–3). Because oxygen homeostasis represents an indispensable organizing principle for human physiology (4), the requirement for oxidative phosphorylation to generate ATP is nevertheless balanced at the cost of oxidative damage to cellular compositions (5). As a tumor expands, vigorous growth of cancer cells creates a hypoxic microenvironment, which, if not alleviated, may restrict tumor growth or even cause cell death (6). *In vivo* studies have shown very convincingly that the areas of tumors stained with hypoxia markers, such as pimonidazole, show reduced proliferation and an increased fraction of apoptotic cells (7). Several *in vitro* studies have also shown that hypoxia induces apoptosis through the activation of HIF-1 (6, 8). However, these studies use a variety of experimental conditions, most often combining reduced oxygen conditions with additional apoptogenic conditions such as glucose and/or serum deprivation. A recent report showed that hypoxia alone (<0.5% oxygen) did not alter the growth of fibroblasts and cancer cells and that anoxia (<0.01% oxygen) induced apoptosis in a HIF-1–independent manner (9), suggesting that hypoxia alone is not sufficient to result in apoptosis. Additionally, areas of tumors that have poor perfusion and reduced delivery of oxygen could also suffer from other kinds of stress, such as low glucose. Glucose, in particular, is an indispensable nutrient under hypoxic conditions because enhanced glucose uptake and glycolysis compensate for the lack of energy production by aerobic metabolism under normoxic conditions (10–12). Indeed, it has been reported that hypoxia causes apoptosis in myocytes only in the absence of glucose and that the presence of as little as 1 mmol/L glucose before exposure to hypoxia prevents apoptosis (13). These findings suggest that glucose-deprived conditions, rather than hypoxic conditions, may be a pivotal contributing factor for the death of cancer cells in the tumor microenvironments, which are always associated with an insufficient supply of glucose, as well as oxygen.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-06-2519

Enhanced anaerobic metabolism, through the enhanced expression of glycolytic enzymes and glucose transporters, is an important hypoxia-induced adaptation response to glucose-deprived conditions. Although under hypoxic glucose-deprived conditions *in vivo*, anaerobic metabolism could function through the enhanced uptake of glucose, which is supplied at very low levels albeit continuously, other mechanisms of tolerance to glucose-deprived conditions might be required. Previous reports showed that hypoxia conferred tolerance to glucose deprivation through the activation of AMP–activated protein kinase (AMPK; refs. 14, 15). Therefore, we hypothesized that glucose deprivation itself might be inducing tolerance to the same glucose deprivation in tumor tissues, where the cells are exposed to hypoxia and glucose deprivation. Cancer cells might be acquiring a tolerance for glucose deficiency during their progression, in addition to the ability to stimulate angiogenesis and glycolysis as hypoxic responses.

To test this hypothesis, we selected a pancreatic cancer cell line as a model because pancreatic cancers are well known to be hypovascular tumors, as determined by means of clinical angiography (16), and sought to identify glucose deprivation–inducible genes by a DNA microarray system. We identified 63 genes whose expressions were enhanced under glucose-deprived conditions at >2-fold higher levels than under normal glucose conditions. Among those genes, we chose asparagine synthetase (*ASNS*) for further study because its mRNA expression levels were the highest among the genes tested by real-time PCR, and its function(s) under glucose-deprived conditions are yet to be determined. Additionally, it has been reported that its expression is enhanced by amino acid deprivation and glucose deprivation and that it is related to drug resistance of leukemia and oncogenesis triggered by mutated p53 (17, 18). ASNS encodes an enzyme that catalyzes the biosynthesis of L-asparagine from L-aspartate in an ATP-dependent reaction for which L-glutamine is the nitrogen source (19). ASNS expression in mammalian tissues is highly regulated by the nutritional status of the cells (20) and is enhanced by glucose deprivation as well as amino acid deprivation (21). The up-regulation of ASNS transcription upon nutrient stress suggests that ASNS may be a critical cellular defense gene against glucose deprivation itself, although the exact roles of overexpressed ASNS under nutrient-deprived conditions have not yet been extensively investigated. To explore this potential defensive feature of ASNS, we examined the roles of ASNS under glucose-deprived conditions in pancreatic cancer cells by silencing ASNS with small interfering RNAs (siRNA) and establishing transfectants overexpressing ASNS.

Materials and Methods

Cell culture. BxPC3, PCI43, and MiaPaCa2 pancreatic cancer cells were cultured in DMEM containing 100 mg/dL glucose (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Japan Bioserum Co., Ltd., Nagoya, Japan), penicillin (25 units/mL), and streptomycin (25 μg/mL). Because normal glucose concentrations in human peripheral blood range between 70 and 200 mg/dL, we used DMEM containing 100 mg/dL glucose. Thus, a concentration of 100 mg/dL is defined as "normal glucose" in our study. As hypoglycemia in human peripheral blood delineates <50 mg/dL glucose, <50 mg/dL glucose in DMEM is defined as "low glucose" in our study. Oxygen concentration in tumor tissues is ~1.25% (about one tenth that in peripheral blood); thus, glucose concentration in tumor tissues could also be estimated to be around one tenth that in peripheral blood. Therefore, the cultures carried out under glucose-deprived conditions were done in glucose-free DMEM, supplemented with 10% FBS (the final concentration of glucose in this medium was 10 mg/dL, as measured in our laboratory), unless otherwise specified. In some experiments, glucose-free DMEM supplemented with 10% FBS and 15 or 40 mg/dL glucose (final concentration of glucose in the medium being 25 or 50 mg/dL) was used. The cells used for the experiments were mainly MiaPaCa2 cells, unless otherwise specified.

Antibodies and chemical reagents. Anti-Flag M2 monoclonal antibody, anti-actin antibody, anti–phospho-stress-activated protein kinase (SAPK)/c-jun NH_2-terminal kinase (JNK; Thr^{183}/Tyr^{185}) antibody, and anti–SAPK/JNK antibody were purchased from Sigma Chemical Co. (St. Louis, MO), Santa Cruz Biotechnology (Santa Cruz, CA), and Cell Signaling Technology (Danvers, MA), respectively. The supernatant from a cloned mouse hybridoma cell line, HL 512 (3G6-2G8), which secretes an antibody [immunoglobulin G (IgG)-2a subclass] that recognizes human ASNS, was used as antihuman ASNS antibody. This was kindly provided by Dr. Sheldon Schuster (Department of Biochemistry, University of Florida, Gainesville, FL). Peroxidase-conjugated goat anti-mouse IgG, peroxidase-conjugated goat anti-rabbit IgG, and peroxidase-conjugated rabbit anti-goat IgG antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Cisplatin [*cis*-diammine-dichloroplatinum (CDDP)] and gemcitabine were kind gifts from Pfizer (Tokyo, Japan) and Eli Lilly (Kobe, Japan), respectively. 5-Fluoruracil (5-FU) was purchased from Calbiochem (La Jolla, CA) whereas paclitaxel, etoposide (VP-16), and carboplatin were kind gifts from Bristol Pharmaceuticals Y.K. (Tokyo, Japan). Compound C, an AMPK inhibitor, was purchased from Calbiochem. The JNK1-3 inhibitor SP600125 was bought from Biomol International (Plymouth Meeting, PA). Hydrogen peroxide (30% solution) was purchased from Wako Pure Chemical Industries (Osaka, Japan).

DNA microarray analysis. Total RNA was extracted with the use of TRIZOL reagent (Life Technologies, Tokyo, Japan) from MiaPaca2 cells that had been incubated for 16 h under glucose-deprived conditions or normal glucose conditions. The glucose concentrations were 10 and 100 mg/dL, respectively. mRNA was purified from total RNA with the use of a QuickPrep mRNA Purification Kit (Amersham Pharmacia Biotech, Tokyo, Japan). The differentially expressed genes were screened using a DNA microarray system (Hokkaido System Science, Sapporo, Japan). We defined the genes that were expressed at >2-fold intensity under glucose-deprived conditions than under normal glucose conditions as possible glucose deprivation–inducible genes in this study.

Sensitivity to anticancer drugs. Sensitivity of tumor cell lines to anticancer drugs was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS) assay according to the manufacturer's instructions (Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI). Briefly, cells were inoculated in 96-well plates (8×10^3 per well) and incubated overnight in a humidified CO_2 incubator (95% air/5% CO_2) in triplicate, to allow the cells to adhere and assume a healthy condition, and then exposed to different doses of anticancer drugs for 48 h under normal glucose conditions. The cells were then incubated for a further 3 h with MTS solution. Subsequently, the absorbance was measured at 490 nm on an ELISA plate reader (model 680, Bio-Rad, Hercules, CA).

Apoptosis assay. Sensitivity of tumor cell lines to apoptosis was determined by two-color fluorescence-activated cell sorting (FACS) analysis using propidium iodide– and FITC-conjugated anti–Annexin V antibody according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). Briefly, after incubating the cells for 48 h under normal glucose conditions or under glucose-deprived conditions, the cells were stained with propidium iodide– and FITC-conjugated anti–Annexin V and then analyzed with a FACSCalibur (Becton Dickinson, Mountain View, CA). Apoptotic cell death was also examined by staining the cells with Hoechst-33342 (Molecular Probes) without fixation. Apoptotic cells were identified by characteristic bright blue fluorescence of nuclei due to condensed or fragmented chromatin. Digital images were acquired with an inverted light microscope (Nikon Eclipse TE300, Nikon Instech Co. Ltd., Kawasaki, Japan) attached to a 100-W halogen lamp source (Nikon Halogen 12V-100W, Nikon Instech) and a charge-coupled device camera (Nikon Coolpix 4500, Nikon).

Western blot analysis. Cells were lysed in cell lysis buffer (Cell Signaling Technology) and whole-cell lysates (40 μg) were run on 10% polyacrylamide gels and electrotransferred to nitrocellulose membranes. The membranes

Table 1. Overexpressed genes under glucose-deprived conditions

Gene		Swiss Prot	Protein Bank
Cell signaling			
EIF2AK3	Eukaryotic initiation factor 2α kinase 3	Q9NZJ5	
PPEF2	Protein phosphatase EF hand 2	O14830	
CPR8	Cell cycle progression 8 protein		AAB69314.1
DNAJC3	DnaJ (Hsp40) homologue subfamily C member 3 (protein kinase inhibitor p58)		BAA74859.1
PRKCZ	Protein kinase Cζ isoform	Q05513	
CDC2	Cell division cycle 2, cyclin-dependent protein kinase	P06493	
SGKL	Serum/glucocorticoid regulated kinase-like		AAF27051.2
ZAP70	ζ-chain-associated 70 kDa protein	P43403	
TOPK	PDZ-binding kinase		BAA99576.1
CDT1	DNA replication factor (double parked)		AAG45181.1
CNK	Cytokine inducible kinase	Q9H4B4	
Transcriptional regulation			
TRPS1	Trichorhinophalangeal syndrome	Q9UHF7	
TGIF	TG-interacting factor	Q15583	
ARNTL	Aryl hydrocarbon receptor nuclear translocator-like		AAC24353.1
RELB	Reticuloendotheliosis viral (v-rel) oncogene related B	Q01201	
PREB	Prolactin regulatory element binding protein	Q9HCU5	
ELF3	E74 like factor 3		AAB58075.1
Enzymes			
ART3	ADP-ribosyltransferase 3	Q13508	
FUT1	Fucosyltransferase 1 [α(1,2) fucosyltransferase, galactoside 2-α-L-fucosyltransferase]	P19526	
NEU1	Neuraminidase 1 (lysosomal neuraminidase, sialidase 1)	Q99519	
GOT1	Soluble glutamate-oxaloacetate transaminase (cytosolic aspartate aminotransferase)	P17174	
ASNS	Asparagine synthetase	P08243	
GFPT1	Glutamine-fructose-6-phosphate transaminase 1	Q06210	
PCK2	Phosphoenolpyruvate carboxykinase 2	Q16822	
CBR3	Carbonyl reductase 3	O75828	
PYCR1	Pyrroline-5-carboxylate reductase 1	P32322	
SARS	Seryl-tRNA synthetase	P49591	
ERO1-L(β)	Endoplasmic reticulum oxidoreductin 1-Lβ		AAF97547.1
NDUFB3	NADH dehydrogenase (ubiquinone) 1β subcomplex 3 (12 kDa B12)	O43676	
RRM2	Ribonucleotide reductase subunit M2	P31350	
B3GNT6	β-1,3-*N*-Acetylglucosaminyltransferase		AAC39538.1
PIGA	GPI GlcNAc transferase a	P37287	
TXNRD1	Thioredoxin reductase 1	Q16881	
Others			
PPIB	Cyclophilin B		AAH20800.1
PRAME	Preferentially expressed antigen of melanoma	P78395	
FKBP2	FK506-binding protein 2 (FKBP13)		AAA36563.1
DFNA5	Deafness autosomal dominant 5	O60443	
RTP801	HIF-1 responsive RTP801		AAL38424.1
SEMG1	Semenogelin I	P04279	
H4FE	H4 histone family member E	P02304	
TFRC	Transferrin receptor	P02786	
GRO2	Macrophage inflammatory protein 2	P19875	
GADD45	DNA damage inducible transcript 1		P24522
TGIF	TG-interacting factor	Q15583	
Transporter proteins			
ACATN	Acetyl-CoA transporter		AAH14416.1
CLGN	Calmegin	O14967	
DNAJB11	DnaJ (Hsp40) homologue subfamily B member 11 (human endoplasmic reticulum–associated DNAJ)	Q9UBS4	
CALR	Calreticulin,	P27797	
CANX	Calnexin	P27824	

(*Continued on the following page*)

Table 1. Overexpressed genes under glucose-deprived conditions (Cont'd)

Gene		Swiss Prot	Protein Bank
SLC3A2	Heavy chain of 4F2 cell-surface antigen	P08195	
UGTREL1	UDP-galactose transporter–related 1		BAA13525.1
KDELR2	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	P33947	
KCNF1	Potassium voltage channel subfamily F1		AAH26110.1
Redox-related proteins			
PDIA4	Protein disulfide isomerase–related protein (calcium-binding protein, intestinal related)	P13667	
TXNIP	Vitamin D3 up-regulated protein-		BAB18859.1
SPS2	Selenophosphate synthetase 2	Q99611	
PDIA6	Protein disulfide isomerase–related protein P5	Q15084	
GRP58	Glucose regulated 58 kDa protein	P30101	
Cytoskeletal proteins			
GFAP	Glial fibrillary acidic protein	P14136	BAB31108.1
GABARAPL3	GABA(A) receptor–associated protein like 3		AAK16237.1
OCLN	Occludin	Q16625	
KIF5C	Kinesin heavy chain 5C	O60282	
ANLN	Aniline		AAF75796.1

were blocked with blocking buffer (5% skimmed milk in 1% Tween-PBS), probed with primary antibodies overnight at 4°C, incubated with a secondary antibodies for 1 h at room temperature, and developed with the use of an enhanced chemiluminescence detection kit (Amersham, Tokyo, Japan).

Real-time PCR. Total RNA was extracted with the use of TRIZOL reagent according to the manufacturer's instructions. Each RNA sample (10 μg) was subjected to cDNA synthesis by means of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Each cDNA (10 ng) was amplified in triplicate with the use of the Quantitect SYBR Green PCR Kit (Qiagen, Hilden, Germany) for 40 cycles (94°C for 15 s, 60°C for 30 s, and 72°C for 1 min) on an ABI PRISM 7900HT Sequence Detection System. The primers used were as follows: ASNS forward primer, CTGCACGCCCTCTATGACA; ASNS reverse primer, TAAAAGGCAGCCAATCCTTCT; β-actin forward primer, TTGCCGACAGGATGCAGAA; β-actin reverse primer, GCCGATCCACACGGAGTACT. Relative mRNA levels were determined by comparing the PCR cycle thresholds between the cDNA of ASNS and that of β-actin.

siRNA preparation and transfection. The antisense sequences of the siRNAs for human ASNS were as follows: AAGAUUGCACACAGAGGUCCA, AAAGUGUUCCUGGGUAGAGAU, and AAAGAAGCCCAAGUACAGUA. These were designed using the *Silencer* siRNA Construction Kit (Ambion, Inc., Austin, TX). A nonsilencing control siRNA was purchased from Qiagen (Valencia, CA). siRNA transfection using 5 nmol/L siRNA was done with Lipofectamine 2000 (Invitrogen) at 30% to 40% cell confluence in an antibiotic-free medium. A cocktail of three siRNAs for human activating transcription factor-4 (ATF-4; siTrio Full Set) was purchased from B-Bridge International (Sunnyvale, CA) and used at 5 nmol/L concentrations. The antisense sequences for these siRNAs were as follows: UUCUCCAACAUCCAAUCUG, AUCUCCUUCAGUGAUAUCC, and UUCUUAUCCAGUUUCUCAC.

Establishment of ASNS transfectants. A cDNA for ASNS was amplified from reverse transcription products of mRNAs purified from MiaPaCa2 cells and cloned into p3XFLAG-cytomegalovirus-14 (CMV-14). PCR primers for the full-length cDNA of ASNS were as follows: forward primer, 5′-GACTGCGGCCGCATAGCTTCATTGCACCA-3′; reverse primer, 5′-CAGTAGATCTGCTTTGACAGCTGACTTG-3′. Plasmids were sequenced with a BigDye Version 3 Sequence Kit (Applied Biosystems, Tokyo, Japan) on an ABI Prism 3100-Avant automated sequencer (Applied Biosystems) according to the manufacturer's protocol. Cloned fragments were ligated into p3XFLAG-CMV-14 (Sigma Chemical). MiaPaCa2 cells were transfected with the expression vector alone (mock transfectant) or the expression vector containing the cloned fragments (ASNS transfectant) with the use of Lipofectamine 2000 (Invitrogen). Transfectants were cloned by a limiting dilution method following selection with G-418 (Sigma; 1,200 μg/mL). The transfectants were then maintained in the presence of 600 μg/mL G-418.

Statistical analysis. Real-time PCR results are represented as mean ± SD, whereas results for MTS assays are represented as means ± 95% confidence intervals (95% CI). Statistical differences among the groups were determined by two-tailed Student's *t* test as for independent samples. Mean values were considered significantly different when $P < 0.05$.

Results

Genes whose expression was enhanced under glucose-deprived conditions. Representative genes whose expression was enhanced under glucose-deprived conditions compared with normal glucose conditions were classified into several categories (Table 1). Our analysis revealed a total of 63 genes up-regulated in MiaPaCa2 cells under glucose-deprived conditions when compared with normal glucose conditions. Because our result comes from a single microarray analysis, conclusions from our analysis cannot be made without further validation of the microarray results by another method, such as real-time PCR. Among the identified genes, *ASNS* was chosen for further study. Recent reports showed that its expression is enhanced by amino acid deprivation and glucose deprivation and that it is related to drug resistance of leukemia and oncogenesis triggered by mutated p53 (17, 18), whereas its function(s) are yet to be determined.

Expression of ASNS under normal glucose/glucose-deprived and hypoxic/normoxic conditions. We examined the expression of ASNS mRNA by real-time PCR in three pancreatic cancer cell lines. In these cell lines, ASNS mRNA was expressed at higher levels following 16-h incubation under glucose-deprived conditions than under normal glucose conditions (Fig. 1*A*). The increase in ASNS mRNA expression was sustained under glucose-deprived conditions in time course experiments (starting from 16 h and lasting up to 48 h; Fig. 1*B*). Various concentrations of glucose, ranging from 50 to 10 mg/dL, increased the expression of ASNS mRNA (Fig. 1*C*). Hypoxia alone increased the expression of ASNS mRNA in one of three cell lines (data not shown). ASNS protein expression was also higher under glucose-deprived conditions than under normal

glucose conditions (Fig. 1*D*). As AMPK has been reported to be an energy sensor (22–24), we then examined the implication of AMPK in the induction of ASNS expression under glucose-deprived conditions. Compound C, an inhibitor of AMPK, has no effect on the enhanced expression of ASNS induced by glucose deprivation (Fig. 1*E*). We then confirmed the role of ATF-4 in the induction of ASNS under glucose-deprived conditions because amino acid deprivation and glucose deprivation have been reported to stimulate the expression of ASNS through the activation of ATF-4 (25, 26). siRNAs for ATF-4 almost completely suppressed the enhanced expression of ASNS under glucose-deprived conditions (Fig. 1*F*). These results suggest that the expression of ASNS under glucose-deprived conditions is induced in an ATF-4–dependent but AMPK-independent manner.

Silencing of ASNS by siRNA sensitizes pancreatic cancer cells to apoptosis. We then examined the roles of ASNS using siRNAs for ASNS. We made three siRNAs, all of which effectively suppressed both the mRNA and protein expressions of ASNS (Fig. 2*A* and *B*). More than 95% of the cells were viable under normal glucose conditions. Although ~50% of the cells treated with a control siRNA were viable under glucose-deprived conditions, the percentages of viable cells treated with two siRNAs

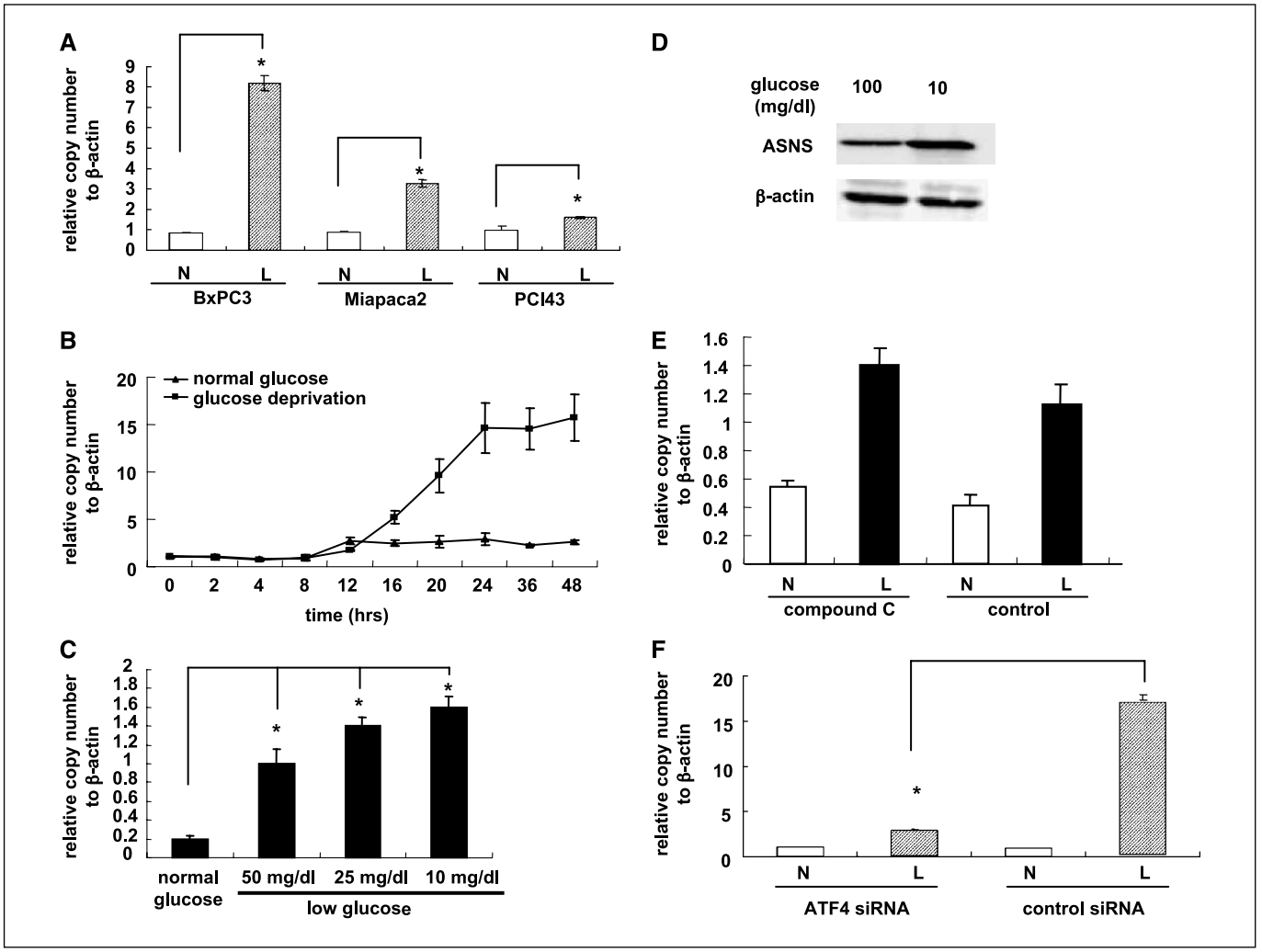

Figure 1. Expression of ASNS under different conditions. *A,* BxPC3, MiaPaCa2, and PCI43 cells were incubated under normal glucose conditions (*N*) and glucose-deprived conditions (*L*). The glucose concentration of the normal glucose group was 100 mg/dL and that of the glucose-deprived group was 10 mg/dL. After incubation for 16 h, the mRNA level of ASNS was analyzed by real-time PCR. β-Actin mRNA was used to standardize the total amount of cDNA. ASNS mRNA expressions were enhanced under low-glucose conditions. Representative results of three independent experiments. *Columns,* mean of triplicate wells; *bars,* SD. *, $P < 0.05$. *B,* MiaPaCa2 cells were incubated under the two different conditions for the indicated times. After incubation, the mRNA level of ASNS was analyzed by real-time PCR. The increase in ASNS mRNA expression was sustained under glucose-deprived conditions in time course experiments (from 16 h up to 48 h). Representative results of three independent experiments. *Points,* mean of triplicate wells; *bars,* SD. *C,* MiaPaCa2 cells were cultured in medium containing varying concentrations of glucose for 16 h and the mRNA level of ASNS was then analyzed by real-time PCR. Varying glucose concentrations (from 50 to 10 mg/dL) enhanced ASNS expression. Representative results of three independent experiments. *Columns,* mean of triplicate wells; *bars,* SD. *, $P < 0.05$. *D,* MiaPaCa2 cells were incubated under the two different conditions for 48 h and the protein levels of ASNS in whole-cell lysates were determined by Western blot analysis. ASNS protein expression was also enhanced under low-glucose conditions. Representative results of three independent experiments. *E,* after preincubation with an AMPK inhibitor, compound C, for 60 min, MiaPaCa2 cells were incubated under normal glucose conditions and glucose-deprived conditions for 16 h. ASNS mRNA was analyzed by real-time PCR. Compound C showed no effect on the expression of ASNS mRNA. Representative results of two independent experiments. *Columns,* mean of triplicate wells; *bars,* SD. *F,* MiaPaca2 cells were transfected with 5 nmol/L siRNA for ATF-4, under normal glucose and glucose-deprived conditions, and total RNA was extracted 48 h after transfection. ASNS mRNA was analyzed by real-time PCR. A mixture of siRNAs for ATF-4 completely suppressed the expression of ASNS mRNA. Representative results of three independent experiments. *Columns,* mean of triplicate wells; *bars,* SD. *, $P < 0.05$.

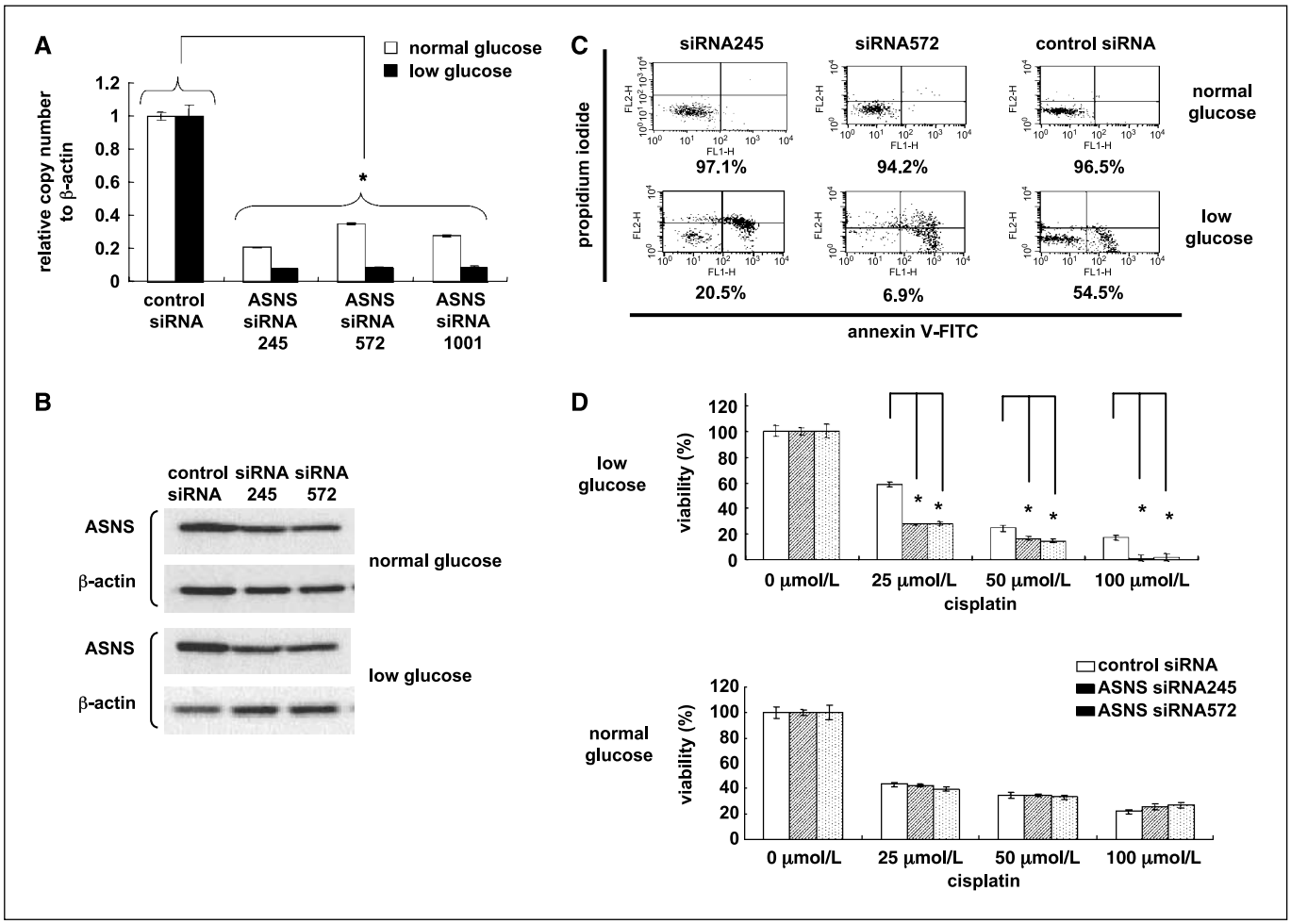

Figure 2. Effects of siRNAs for ASNS on the sensitivity to apoptosis. *A,* MiaPaca2 cells were transfected with 5 nmol/L siRNA for ASNS, under normal glucose and glucose-deprived conditions, and total RNA was extracted 48 h after transfection. ASNS mRNAs were analyzed by real-time PCR. The relative copy number to β-actin in the cells treated with control siRNA, under normal or low-glucose conditions, was artificially defined as one. All siRNAs for ASNS efficiently suppressed the expression of ASNS mRNA. Representative results of three independent experiments. *Columns,* mean of triplicate wells; *bars,* SD. *, $P < 0.05$. *B,* MiaPaca2 cells were transfected with 5 nmol/L siRNA for ASNS, under normal glucose and glucose-deprived conditions, and whole-cell lysates were prepared 48 h after transfection. Protein levels of ASNS were analyzed by Western blot analysis. Two siRNAs for ASNS efficiently suppressed the expression of ASNS protein. Representative results of three independent experiments. *C,* MiaPaca2 cells were transfected with 5 nmol/L siRNA for ASNS under normal glucose and glucose-deprived conditions for 48 h. Viable cells were analyzed by FACS two-color analysis using propidium iodide– and FITC-conjugated Annexin V. siRNAs for ASNS decreased the percentages of viable cells under low-glucose conditions. Representative results of four independent experiments. *D,* MiaPaCa2 cells, transfected with 5 nmol/L siRNA for ASNS, were cultured under normal glucose and glucose-deprived conditions with indicated concentrations of CDDP for 48 h. Viable cells were analyzed by a colorimetric MTS assay. siRNAs for ASNS decreased the percentages of viable cells following CDDP treatment only under glucose-deprived conditions. Representative results of three independent experiments. *Columns,* mean of triplicate wells; *bars,* 95% CI. *, $P < 0.05$.

for ASNS (siRNA245 and siRNA572) decreased to ~20% and 7% under glucose-deprived conditions (Fig. 2*C*). These results suggest that overexpression of ASNS under glucose-deprived conditions plays an important role in the protection of cancer cells from apoptosis induced by glucose-deprived conditions. In addition to apoptosis induced by glucose deprivation, we examined the effects of silencing *ASNS* during treatment with an anticancer drug, CDDP. The cells treated with siRNAs for ASNS under glucose-deprived conditions were also more sensitive to apoptosis induced by CDDP, which, by itself, did not induce the expression of ASNS mRNA (Supplementary Fig. S1*A*), than those treated with a control siRNA (Fig. 2*D, top*). Under normal glucose conditions, siRNAs for ASNS showed no effect on the sensitivity to apoptosis induced by CDDP (Fig. 2*D, bottom*). These results suggest that glucose deprivation induced resistance to CDDP through the enhanced expression of ASNS.

Overexpression of ASNS protects cancer cells from apoptosis. We then established MiaPaCa2 cell lines overexpressing ASNS constitutively. Before cloning, we examined the sensitivity of the ASNS transfectants to apoptosis induced by glucose deprivation and confirmed that they were significantly more resistant to apoptosis than the mock transfectant (Fig. 3*A*). Staining with Hoechst-33342 showed that more than half of the mock-transfected cells cultured under low-glucose conditions showed bright fluorescence, representing apoptotic cells, whereas nuclei of the ASNS-transfected cells displayed only dim staining, except for the small fraction (Fig. 3*B*). Two transfectant clones expressing Flag-tagged ASNS were selected for further experiments (Fig. 3*C*). These two ASNS transfectant clones were also more resistant to apoptosis induced by glucose deprivation than the mock-transfectant (Fig. 3*D*). Viable cells were examined by means of the MTS assay after incubating mock-transfected cells and ASNS-transfected cells

under low-glucose conditions for up to 48 h (Supplementary Fig. S1*B*). After 24 and 48 h of incubation, ~100% and 50%, respectively, of the ASNS-transfected cells were viable, whereas the percentages of the viable mock-transfected cells were significantly lower than those of the ASNS-transfected cells. We then examined the expression levels of bcl-2 family proteins (bcl-2, bcl-xL, and Bax) in the transfectants, but we could not find any difference in bcl-2 family protein expressions between the mock transfectant and the ASNS transfectants (data not shown). We proceeded to examine the activation status of stress-inducible kinases such as JNK/SAPK. We found that glucose deprivation clearly activated JNK/SAPK in the mock transfectant but not in the ASNS transfectant (clone 7; Fig. 3*E*). Moreover, an inhibitor of JNK increased the percentage of viable MiaPaCa2 parent cells cultured under glucose-deprived conditions for 24 h in a dose-dependent manner (Fig. 3*F*). These results suggest that overexpression of ASNS, induced by glucose deprivation, plays an important role in the survival of pancreatic cancer cells through the suppression of JNK activation under glucose-deprived conditions. Furthermore, the sensitivity of uncloned ASNS transfectants to apoptosis induced by CDDP, under normal glucose conditions, was significantly lower than that of the mock transfectant (Supplementary Fig. S1*C*). When

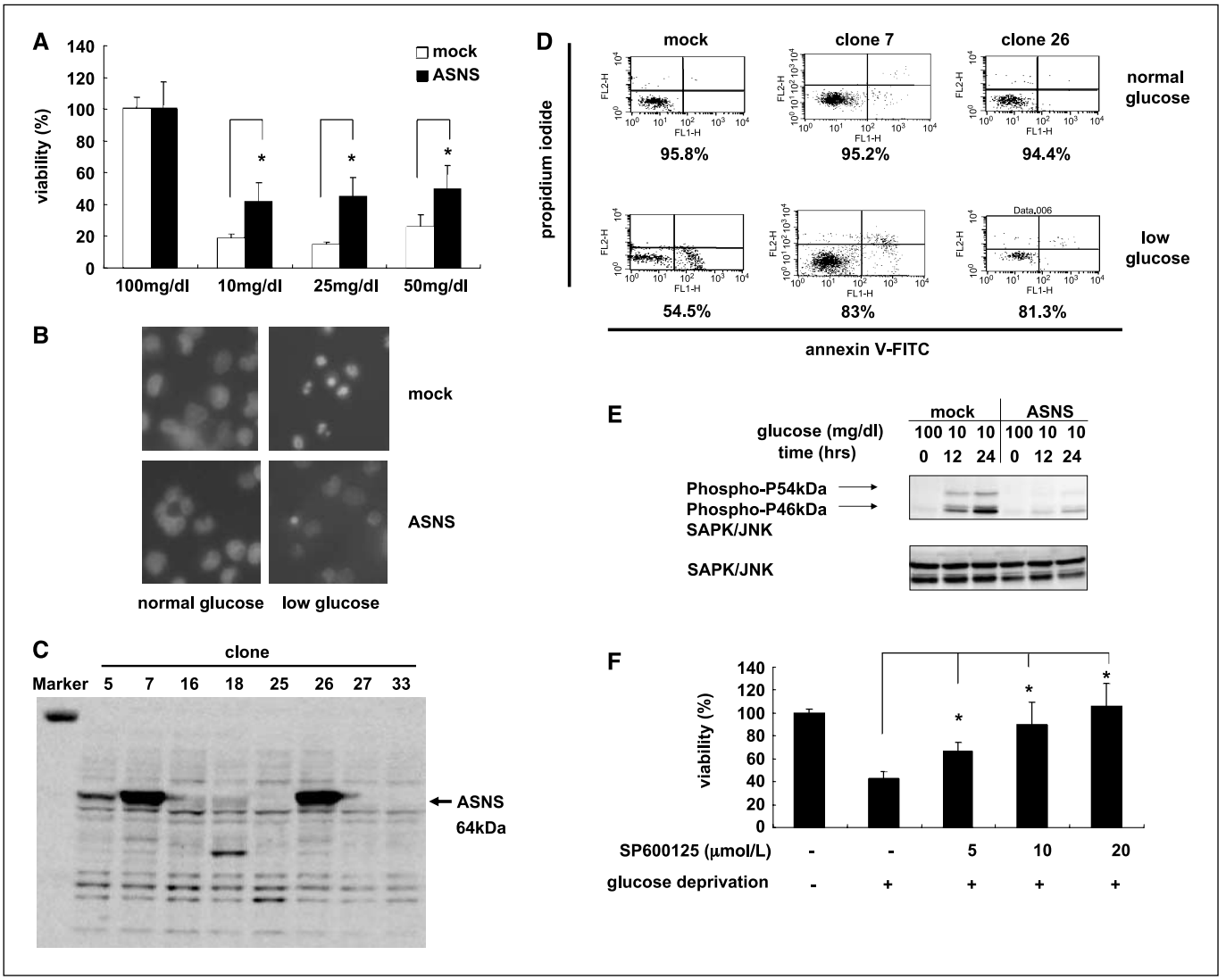

Figure 3. Establishment of ASNS transfectants. *A,* the uncloned ASNS-transfected and mock-transfected cells were cultured under normal glucose and varying glucose concentrations for 48 h. Viable cells were analyzed by means of a colorimetric MTS assay. The uncloned ASNS transfectant is more resistant to apoptosis induced by glucose deprivation than the mock transfectant. Representative results of three independent experiments. *Columns,* mean of triplicate wells; *bars,* 95% CI. *, $P < 0.05$. *B,* digital images acquired by fluorescence microscopy. Staining with Hoechst-33342 reveals that more than half of the mock-transfected cells cultured under low-glucose conditions show bright fluorescence (representing apoptosis), whereas nuclei of the ASNS-transfected cells display only dim staining, except for a small fraction. *C,* MiaPaCa2 cells were transfected with the p3XFLAG-CMV™-14 expression vector with the use of Lipofectamine 2000. Expression levels of ASNS protein in the transfectants are shown. Clone 7 and clone 26 were chosen for further experiments. *D,* the transfectants (ASNS-transfected clones 7 and 26 and a mock transfectant) were cultured under normal glucose and glucose-deprived conditions for 48 h. Viable cells were analyzed by FACS two-color analysis using propidium iodide– and FITC-conjugated Annexin V. The ASNS transfectants are resistant to apoptosis induced by glucose deprivation. *E,* JNK/SAPK activation by low glucose in mock and ASNS transfectants is shown by Western blotting. ASNS clearly inhibits JNK/SAPK activation under glucose-deprived conditions. *F,* MiaPaCa2 parent cells were incubated under glucose-deprived conditions for 24 h in the presence (+) or absence (−) of SP600125, an inhibitor of JNK. After incubation, viable cells were analyzed by a colorimetric MTS assay. SP600125 increased the number of viable cells. *Columns,* mean of three different experiments; *bars,* 95% CI. Viability is compared with the cells cultured without SP600125 (−), under normal glucose conditions (−). *, $P < 0.05$.

we examined the sensitivity of the ASNS-transfected clones to other anticancer drugs, the ASNS transfectants were more resistant to CDDP and carboplatin but not to 5-FU, VP-16, paclitaxel, or gemcitabine than the mock transfectant (Fig. 4*A–F*).

Sensitivity of MiaPaCa2 parent cells to CDDP in the presence of asparagine. Because overexpression of ASNS induces the accumulation of asparagine in the cells, we then examined the effect of the addition of L-asparagine into the medium. Higher concentrations of asparagine in the medium (up to 20 mmol/L) showed no effect on the sensitivity of MiaPaCa2 parent cells to CDDP (Fig. 5*A*).

Phosphorylation of JNK in ASNS transfectants treated with CDDP or 5-FU. We also examined the activation of JNK/SAPK in the cells treated with CDDP and 5-FU. We found that CDDP, but not 5-FU, induced a significant activation of JNK/SAPK in the mock transfectant (Fig. 5*B*). Overexpression of ASNS suppressed the activation of JNK/SAPK induced by CDDP in the ASNS transfectant (clone 7; Fig. 5*B*). We then examined the effects of a JNK inhibitor on the sensitivity of the MiaPaCa2 parent cells to CDDP to mimic the inhibiting effects of ASNS on JNK. As predicted, treatment of the cells with the inhibitor of JNK for 48 h increased the percentage of viable MiaPaCa2 parent cells treated with CDDP compared with the control. However, it did not show any effect on the numbers of viable cells treated with 5-FU (Fig. 5*C*).

Discussion

Here, we clearly show that glucose deprivation induces the expression of ASNS, in accordance with previous reports (10, 27), and that the enhanced expression of ASNS protects pancreatic cancer cells from apoptosis induced by glucose deprivation through the suppression of JNK/SAPK activation. These results

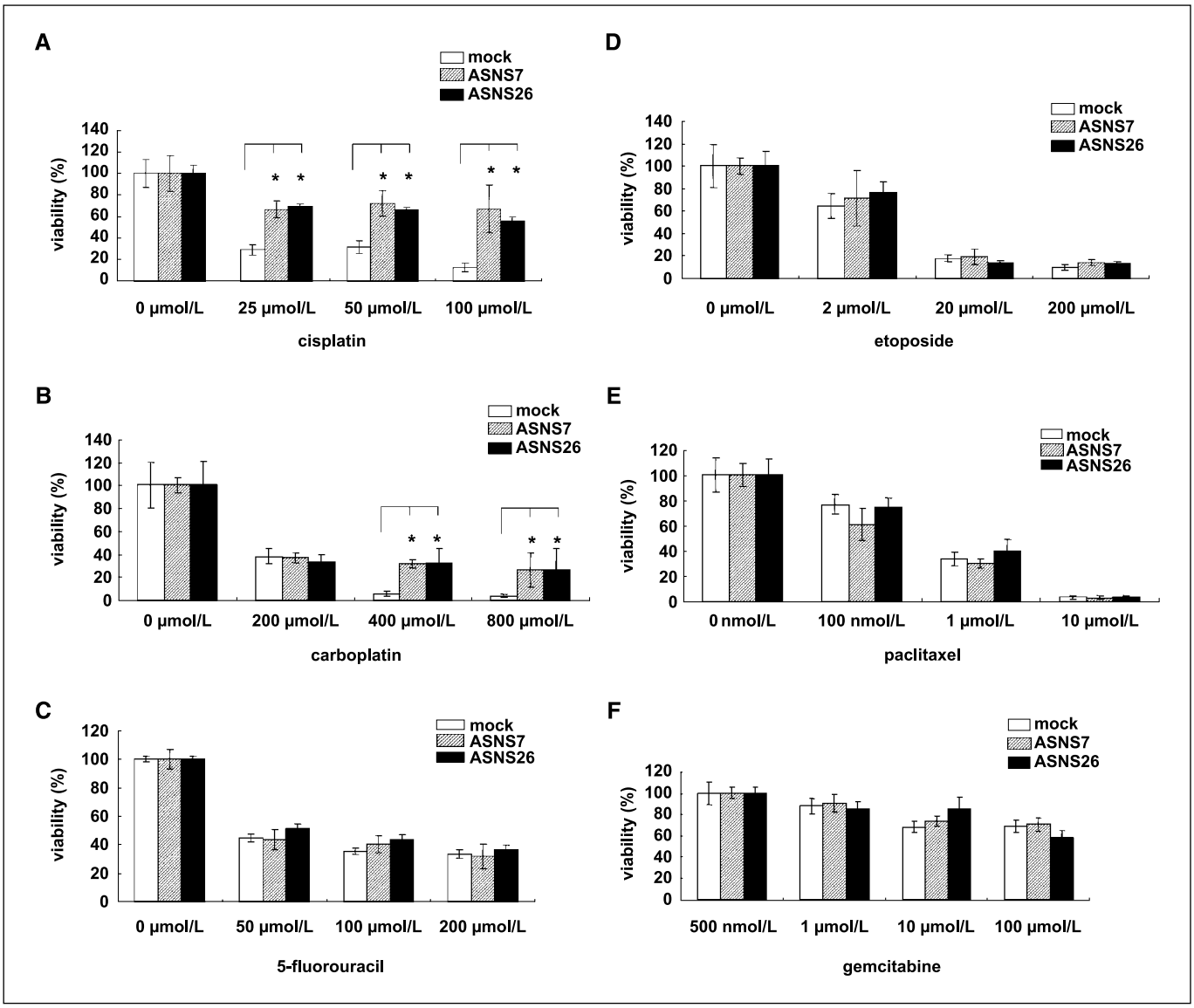

Figure 4. Sensitivity of ASNS transfectants to several anticancer drugs. ASNS transfectants were incubated under normal glucose conditions with the indicated concentrations of CDDP (*A*), carboplatin (*B*), 5-FU (*C*), VP-16 (*D*), paclitaxel (*E*), and gemcitabine (*F*) for 48 h. After incubation, viable cells were analyzed by a colorimetric MTS assay. The two transfectant clones are resistant to both CDDP and carboplatin but not to 5-FU, VP-16, paclitaxel, or gemcitabine. Representative results of three independent experiments. *Columns,* mean of triplicate wells; *bars,* 95% CI. *, $P < 0.05$.

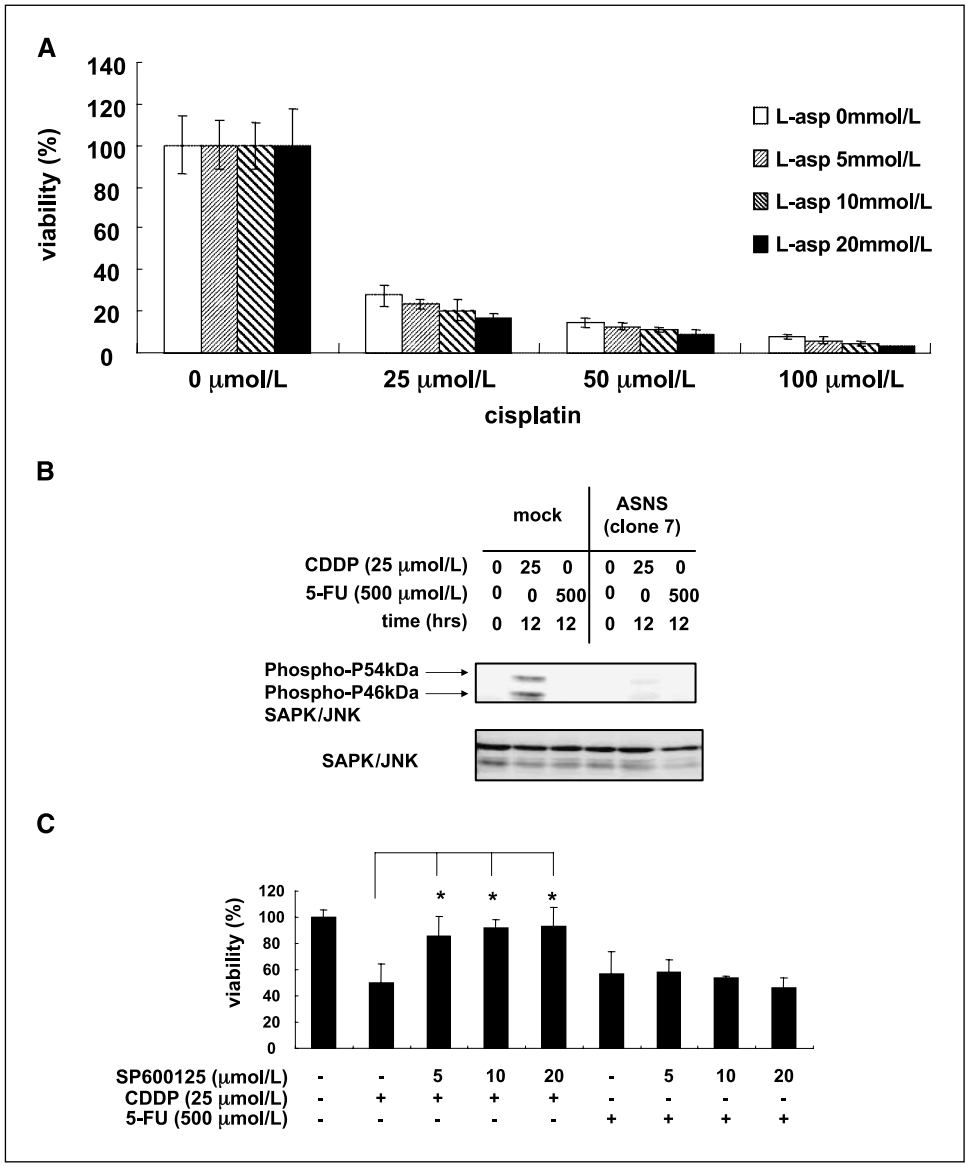

Figure 5. Roles of asparagine and JNK/SAPK in CDDP resistance. *A,* asparagine added in the culture medium up to 20 mmol/L had no effect on the MiaPaCa2 cell sensitivity to CDDP following 48 h of incubation under normal glucose conditions. Viable cells were analyzed by a colorimetric MTS assay. Representative results of three independent experiments. *Columns,* mean of triplicate wells; *bars,* 95% CI. *B,* JNK/SAPK activation induced by CDDP and 5-FU. CDDP but not 5-FU clearly activated JNK/SAPK after 12-h incubation under normal glucose conditions. The activation of JNK/SAPK was suppressed in the ASNS transfectant. Representative results of three independent experiments. *C,* MiaPaCa2 parent cells were cultured under normal glucose conditions for 48 h in the presence (+) or absence (−) of CDDP, 5-FU, and SP600125 (an inhibitor of JNK), as illustrated. After incubation, viable cells were analyzed by a colorimetric MTS assay. Representative results of three independent experiments. *Columns,* mean of triplicate wells; *bars,* 95% CI. *, $P < 0.05$.

show for the first time that ASNS, induced by glucose deprivation, is implicated in adaptation responses to the same glucose-deprived conditions, frequently observed in tumor tissues. Furthermore, we show that the enhanced expression of ASNS protects pancreatic cancer cells from apoptosis induced by CDDP and that this resistance to CDDP is also mediated through the suppression of JNK/SAPK activation. As recent studies showed that potent ASNS inhibitors may have clinical usefulness in treating childhood acute lymphoblastic leukemia, which overexpresses ASNS (17), we envisage that inhibitors of ASNS may also become a beneficial tool for treating pancreatic cancers.

ASNS encodes an enzyme that catalyzes the glutamine- and ATP-dependent conversion of aspartic acid to asparagine (19). The expression of ASNS is enhanced by amino acid deprivation, and this regulation is transcriptional in nature (20). A recent report showed that the human *ASNS* gene is also activated when cells are incubated in the absence of glucose (21). The cellular significance of ASNS overexpression induced by glucose deprivation is not yet fully understood, although it is reported that ASNS may be a serum marker for neoplasias (28) and is related with cell cycle regulation (29) and resistance to apoptosis induced by L-asparaginase (30). As the addition of L-asparagine in the culture medium had no effect on the resistance to CDDP, it seems unlikely that ASNS may induce resistance to apoptosis through the accumulation of L-asparagine, produced by ASNS. Our work reveals that glucose deprivation, CDDP, and carboplatin, but not 5-FU, activated JNK/SAPK and that the overexpression of ASNS suppressed this activation. These results suggest that glucose deprivation and CDDP may induce apoptosis, at least partially, through the activation of JNK/SAPK and that the overexpression of ASNS may inhibit apoptosis through the suppression of this JNK/SAPK activation. Unlike our results, several reports showed that 5-FU and other anticancer drugs also induced apoptosis through the activation of JNK/SAPK (31–34). Contrastingly, the genetic removal of SEK-1, an immediate upstream activator kinase of JNK, promoted apoptosis of hepatocytes and T cells (35, 36), which proposes that JNK has a dual role in cell survival. These contradictory results suggest that the role of the activation of JNK in cell survival is still unclear.

A definite mechanism for the role of ASNS in cell survival also remains to be determined.

Our results show that ATF-4, but not AMPK, is involved in the induction of ASNS expression by glucose deprivation. Glucose deprivation induces two major cellular response pathways: an unfolded protein response pathway and an AMPK-dependent signaling pathway (22, 23, 37, 38). AMPK is an energy sensor that regulates the energy balance within the cell by means of switching off ATP-consuming pathways and switching on ATP-generating pathways (22). Because it has been reported that glucose deprivation increased the mRNA level of some hypoxia-inducible genes, such as vascular endothelial growth factor (*VEGF*), glucose-transporter 1 (*Glut-1*), and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (*PKFKB3*; ref. 39), we assumed that AMPK was involved in enhancing the expression of ASNS mRNA under glucose-deprived conditions. However, our result clearly shows that AMPK is not involved in the signal transduction pathway for ASNS induction. Our finding on ATF-4 is consistent with previous reports showing that ATF-4 is one of the mediators of the nutrient-sensing response pathway, also known as the unfolded protein response pathway, which activates the human *ASNS* gene (25, 26). It is well known that many disturbances in the normal functions of the endoplasmic reticulum lead to the unfolded protein response pathway, which is aimed initially at compensating for damage but can eventually trigger cell death if endoplasmic reticulum dysfunction is severe and prolonged (37). Hypoxia and CDDP, as well as glucose deprivation, also lead to endoplasmic reticulum stress and then the unfolded protein response pathway (37, 40, 41). In our study, hypoxia induced the expression of ASNS mRNA in only one of the three cell lines tested (data not shown). Furthermore, CDDP, which has been reported to induce endoplasmic reticulum stress, did not increase the expression of ASNS mRNA (Supplementary Fig. S1*A*). All these results suggest that ATF-4 is an essential transcription factor for enhanced expression of ASNS under glucose-deprived conditions and that glucose deprivation may activate an ATF-4 pathway independently of endoplasmic reticulum stress.

Because tumor tissues are exposed to glucose-deprived conditions, as well as hypoxic conditions, adaptation responses against hypoxia through the activation of the HIF-1 pathway are not enough for the survival of solid tumor cells *in vivo*. Recently, we have reported that transforming growth factor-β was involved in the tolerance to glucose starvation under hypoxic conditions (15). This finding indicates that hypoxia induces the adaptation responses to glucose deprivation under hypoxic, glucose-deprived conditions. Our question in this study was whether glucose deprivation itself induces any adaptation responses to hypoxic, glucose-deprived conditions. Our present results clearly indicate that one effect of glucose deprivation is the induction of adaptation responses to apoptosis brought about by the same glucose deprivation. We now propose that glucose deprivation itself induces the adaptation responses of cancer cells to the tumor microenvironment other than the HIF-1 pathway activated by hypoxia.

Collectively, our results provide evidence that glucose deprivation induces the overexpression of ASNS in pancreatic cancer cells, which in turn leads to resistance to apoptosis. Consequently, ASNS overexpression suppresses the activation of JNK/SAPK, thus inhibiting death signal transduction. Hence, ASNS may very well be an important therapeutic target for pancreatic cancers, as well as acute lymphoid leukemias.

Acknowledgments

Received 7/11/2006; revised 11/27/2006; accepted 1/19/2007.

Grant support: Cancer Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology (M. Kobayashi).

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We thank Dr. Jun-ichi Hamada (Division of Cancer-related Genes, Institute for Genetic Medicine, Sapporo, Japan) and Dr. Hiroshi Ishikura (The First Department of Pathology, Hokkaido University School of Medicine, Sapporo, Japan) for providing us with the cell lines, Dr. Sheldon Schuster (Department of Biochemistry, University of Florida College of Medicine) for providing us with the anti-ASNS antibody, and M. Yanome for her valuable assistance in the preparation of the manuscript.

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Enhanced Expression of Asparagine Synthetase under Glucose-Deprived Conditions Protects Pancreatic Cancer Cells from Apoptosis Induced by Glucose Deprivation and Cisplatin

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Cancer Res 2007;67:3345-3355.