Role of ASC in hypoxia-mediated cell death in pancreatic cancer

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Abstract. ASC, an apoptosis-associated speck-like protein, can regulate apoptosis in response to various types of cell death stimuli. In this study, we investigated the role of ASC in hypoxia-mediated cell death in pancreatic cancer. ASC was inducible under a 1% O_2 hypoxic condition in pancreatic cancer cells, which was HIF1α-dependent but p53-independent. Two representative chemotherapeutic agents for pancreatic cancer, 5-fluorouracil and gemcitabine, promoted cell death in a p53-dependent manner; however, 1% hypoxia caused chemoresistance to these drugs. Western blot analysis of this condition showed that expression of Bax, a pro-apoptotic gene, decreased, while the anti-apoptotic genes IAP-2 and survivin increased. These results suggest that, although hypoxia induces both pro-apoptotic and anti-apoptotic genes, the total balance seems to be anti-apoptotic dominant, which might explain chemoresistance in pancreatic cancer. To overcome this anti-apoptotic dominant condition, we infected adenovirus-expressing ASC into pancreatic cancer cells. The expressed ASC induced cell death even under 20% normoxia, and was enhanced by hypoxia. Our data demonstrate the possible mechanism of chemoresistance under hypoxia in pancreatic cancer cells, thereby suggesting the potential use of ASC as a new treatment strategy for pancreatic cancer.

Introduction

Despite recent advances in surgical techniques and the discovery of new chemotherapeutic agents, pancreatic cancer remains difficult to cure (1). One of the reasons for its poor patient prognosis is considered to be the hypoxic conditions associated with this disease due to its hypovascularity; many previous studies have demonstrated that hypoxia causes chemoresistance and accelerates tumor progression, invasiveness and metastasis in pancreatic cancer (2-8).

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ASC, an apoptosis-associated speck-like protein, is a unique protein which consists of two protein-protein interaction domains, an amino-terminal pyrin domain and a caspase recruitment domain in the carboxyl terminal (9). We previously demonstrated that ASC is a target gene of p53 and regulates the p53-Bax mitochondrial apoptotic pathway induced by many chemotherapeutic drugs (10,11). As well, we showed that ASC can regulate the p53-independent apoptotic pathway by such agents as tumor necrosis factor (TNF)-α or indomethacin (10). Others have shown that ASC affects nuclear factor κ-B (NF-κB) function through its binding to IKK in response to pro-inflammatory stimulation (12). Therefore, ASC can regulate cell death signaling in response to various types of stimuli, and it is possible that ASC may be a regulator of hypoxia-mediated cell death signaling as well. In this study, we investigated the role of ASC in hypoxia-mediated cell death using pancreatic cancer cells.

Materials and methods

Cell lines and constructs. Seven pancreatic cancer cell lines, PK-1, KP-4, PK-8, PK-9, PK-59, Miapaka and KLM-1, were purchased from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and from the Riken Cell Bank (Ibaragi, Japan). The cells were cultured in RPMI-1640 medium (Sigma) containing 10% fetal bovine serum (Sigma) and 100 μg/ml kanamycin (Meiji, Tokyo, Japan), and were incubated at 37˚C in a humidified atmosphere containing 20% O_2 and 5% CO_2 in air. Thereafter, some cells were moved to a hypoxic chamber (Astec, Fukuoka, Japan) containing 1% O_2, 5% CO_2 and 94% N_2 at 37˚C. Recombinant adenovirus-expressing myc-tagged ASC (Ad-ASC) and green fluorescent protein (Ad-GFP) as a control were previously described (10). Sense and antisense oligonucleotides corresponding to the following cDNA sequences were purchased from iGene (Ibaragi, Japan): CCAGCAGACUCAAAUACAAGAACCUAG for hypoxia-inducible factor-1α (HIF-1α) and GAGAGCUGAUUUACG GAUGUAGAAGAG for scramble (control). The cells were grown to 50% confluency in a 6-well plate and infected with adenovirus at a multiplicity of infection of 30, or transfected with small interference RNA (siRNA) using a Microporator (Digital Bio Technology Co., Suwon, Korea) according to the manufacturer's instructions in the presence of siRNA. Regarding drug treatment, the cells were exposed to 5-fluorouracil

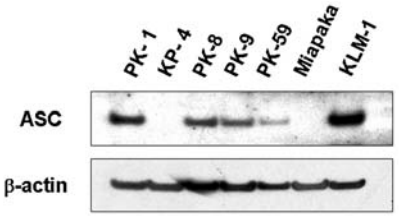

Figure 1. The expression of the protein level of ASC in pancreatic cancer cells. Seven pancreatic cancer cell lines were lysed in lysis buffer and subjected to Western blot analysis using antibodies against ASC and ß-actin (control).

(5-FU, Sigma) at a concentration of 600 nM or gemcitabine (GEM, Eli Lilly) at 1 μM for 24 h.

Western blot analysis. Cells were lysed in lysis buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% NP-40 and 1 mM PMSF) including Protease Inhibitor Cocktail (1:200 dilution, Sigma). Equal amounts of total cellular proteins per sample were subjected to SDS-PAGE on a 10% Bis-Tris gel (Invitrogen) and then transferred to a nitrocellulose membrane (Invitrogen). Primary antibodies for immunoblotting included anti-ASC (Chemicon), -p53 (DO-1; Santa Cruz), -HIF-1α (R2; Novus), -IAP-2 (H-85, Santa Cruz), -survivin (FL142, Santa Cruz), -Bax (N-20, Santa Cruz) and -ß-actin (Sigma). Secondary antibodies were goat anti-rabbit and rabbit anti-mouse conjugated with horseradish peroxidase (Santa Cruz). Bands were detected using the ECL chemiluminescence detection method (Amersham).

Cell death analysis (Trypan blue exclusion test). Cells were pelleted and washed with phosphate-buffered saline. Trypan blue (Gibco) was added to the cell pellets, and stained cells were counted as dead. Experiments were repeated 3 times, and values were expressed as the mean ± standard deviation.

Results

Expression of ASC in pancreatic cancer cells. Seven pancreatic cancer cell lines were lysed in lysis buffer and subsequently subjected to Western blot analysis. Fig. 1 shows that ASC expression was observed in 5/7 cell lines (71%). Of these, PK-1 was mainly used in our experiments since ASC, wild-type p53, HIF-1α and other apoptosis-associated genes such as Bax, IAP-2 and survivin were all well preserved.

ASC induction under hypoxia. PK-1 and PK-8 (p53-null) were incubated in 1% O_2 and collected at the indicated times. Fig. 2A shows ASC induction under hypoxia in PK-1 cells. HIF-1α was also induced by hypoxia, while the protein level of p53 was no different from the level observed under normoxia. When using p53-null PK-8 cells, ASC induction was also noted, with a parallel change in HIF-1α and its target gene IAP-2 (Fig. 2B). To ascertain whether ASC induction was HIF-1α-dependent or not, siRNA against HIF-1α was transfected into PK-1 cells and incubated under hypoxia. This siRNA construct almost totally suppressed the expression of HIF-1α under hypoxia, and the expression levels of ASC and IAP-2 also decreased in comparison to untransfected cells under hypoxia (Fig. 2C). These data suggest that ASC induction under hypoxia is p53-independent but HIF-1α-dependent.

Effect of hypoxia on chemosensitivity. To determine the effect of hypoxia on chemosensitivity in pancreatic cancer cells, PK-1 cells were treated by two representative chemotherapeutic agents for pancreatic cancer, GEM and 5-FU, with or without 1% O_2 hypoxia for 24 h. Fig. 3A shows that GEM induced cell death through the p53-Bax pathway, and that a 1% O_2 hypoxic condition inhibited such chemosensitivity. Notably, Western blot analysis demonstrated that hypoxia suppressed the expression level of Bax, known as a key regulator of the p53-mitochondrial cell death signaling pathway, but increased the protein levels of anti-apoptotic molecules such as IAP-2 and survivin (Fig. 3B). The same phenomenon was observed when applying 5-FU (Fig. 3C and D). These results indicate that although hypoxia induces both a pro-apoptotic gene (ASC induction) and an anti-apoptotic condition (Bax repression and inductions of IAP-2 and survivin), the total balance seems to be anti-apoptotic dominant.

Effect of expressed ASC on hypoxia-mediated cell death. PK-1 cells were infected with Ad-ASC or Ad-GFP (as a control), and were incubated 24 h after infection with or without hypoxia, then collected at the indicated times to determine cell death

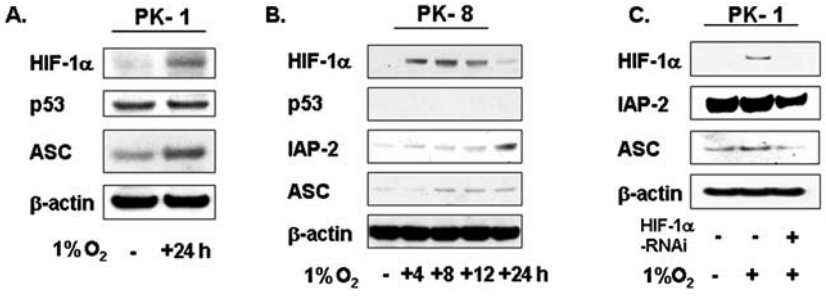

Figure 2. (A) The induction of ASC under hypoxia. PK-1 cells were incubated in 1% O_2 for 24 h and subjected to Western blot analysis using antibodies against HIF-1α, p53, ASC and ß-actin. (B) The p53-independent induction of ASC under hypoxia. PK-8 cells (p53-null) were incubated in 1% O_2 and collected at the indicated times. Next, the cells were subjected to Western blot analysis using anti-HIF-1α, -p53, -ASC, -IAP-2 and -ß-actin antibodies. (C) ASC induction under hypoxia is HIF-1α-dependent. PK-1 cells were transfected with siRNA against HIF-1α or scramble siRNA (control). Twenty-four hours after transfection, the cells were incubated under 20% O_2 normoxia or 1% O_2 hypoxia. Next, the cells were collected and subjected to Western blot analysis.

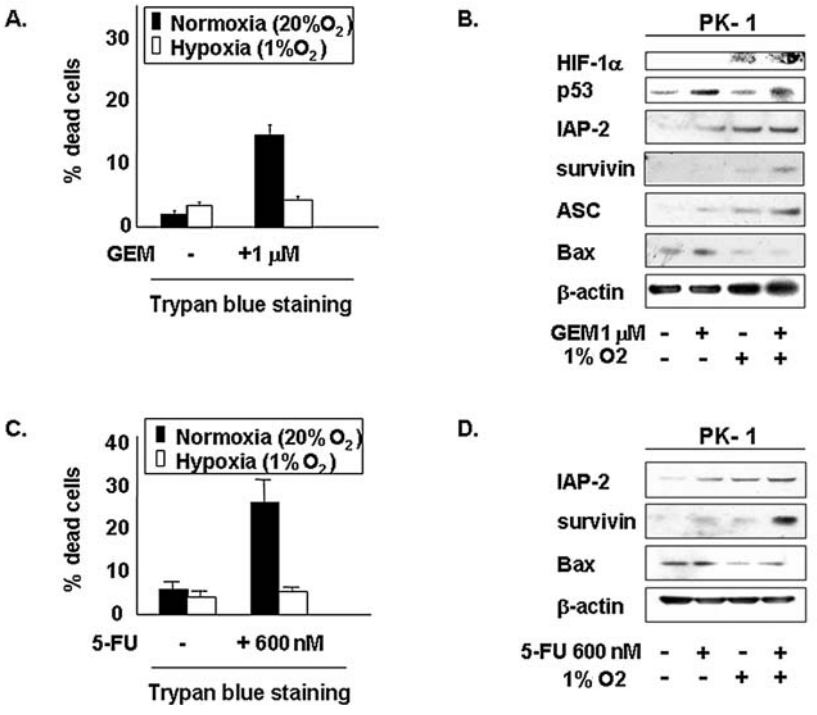

Figure 3. Hypoxia induces chemoresistance. (A) PK-1 cells were treated with 1 μM of gemcitabine (GEM) with or without hypoxia for 24 h. (B) Next the cells were subjected to Western blot analysis and Trypan blue exclusion analyses. The primary antibodies were anti-HIF-1α, -p53, -IAP-2, -survivin, -ASC, -Bax and -ß-actin. (C and D) PK-1 cells were treated with 600 nM of 5-fluorouracil (5-FU) with or without hypoxia for 24 h. Thereafter, the cells were subjected to Trypan blue staining analyses (C) and Western blot analysis (D).

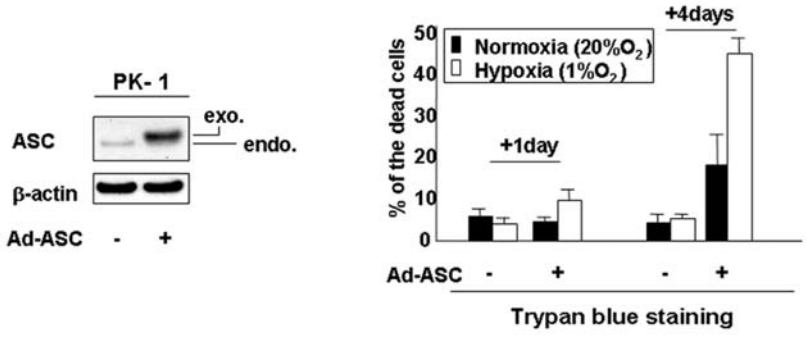

Figure 4. Hypoxia enhances ASC-mediated cell death. PK-1 cells were infected with adenovirus-expressing ASC or GFP (control). Twenty-four hours after infection, the cells were incubated with or without hypoxia and were subsequently collected at the indicated times to undergo both Western blotting and Trypan blue staining analyses.

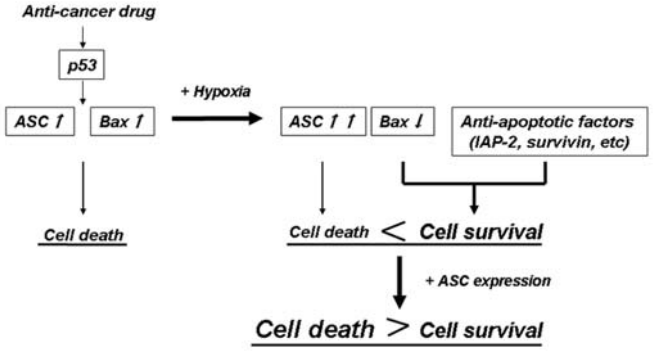

Figure 5. The possible mechanism of hypoxia-induced chemoresistance and the effect of ASC on hypoxia-mediated cell death in pancreatic cancer cells.

using Trypan blue exclusion analysis. Fig. 4 showed that the expressed ASC induced 20% cell death even under normoxia, and that hypoxia increased this ASC-induced cell death to >40% 4 days after hypoxic incubation.

Discussion

In the present study, we found ASC to be a novel hypoxia-inducible gene expressed in a HIF-1α-dependent manner. Since the protein level of Bax, a collaborator of ASC, decreases under 1% O_2 hypoxia, a physiological level of ASC might not be able to promote cell death. However, ectopically-expressed ASC induced cell death in pancreatic cancer cells and was enhanced by hypoxia. Fig. 5 shows the possible mechanism controlling the role of ASC under hypoxia-mediated cell death.

Our previous study demonstrated that ASC binds to Bax at the pyrin domain and regulates the translocation of Bax to the mitochondria for the release of cytochrome c, which is a critical step in the intrinsic apoptotic pathway (10). Bax is also a direct target gene of p53, like ASC, thus p53-mediated inductions of ASC and Bax in response to chemotherapeutic drugs are essential elements of mitochondrial cell death signaling (10,13,14). Notably, our present study showed differential regulation of these two genes under 1% O_2 hypoxia: ASC induction and Bax repression. Yokoi and Fidler demonstrated that hypoxia increases the resistance of pancreatic cancer to GEM-induced cell death through the activation of the phosphatidylinositol 3'-kinase (PI3K)/Akt/NF-κB signaling pathway (6). They also showed that the simultaneous inhibition of growth factor-associated receptors, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor and platelet-derived growth factor receptor (PDGFR), increases the chemosensitivity of pancreatic cancer to GEM (7). In addition to the contribution of growth factors to chemoresistance in pancreatic cancer, other researchers have shown the effect of apoptosis-related genes on hypoxia-mediated chemoresistance or tumor growth using various types of cancer. Liu *et al* demonstrated the relationship between hypoxia-induced chemoresistance in gastric cancer and Bcl-2 family proteins. In that study, the expression of anti-apoptotic gene Bcl-2 and the suppression of pro-apoptotic Bax were possible causes (15). Fei *et al* also showed the suppression of Bax under hypoxia in sarcoma cells (16). These findings support our observations, and Bax repression might be one of the critical reasons for hypoxia-induced chemoresistance in pancreatic cancer.

Although p53 is well known to be a tumor suppressor gene (13,14), it also induces pro-apoptotic as well as anti-apoptotic genes to regulate the balance of cellular death in response to anti-cancer drugs (17,18). It has also been reported that HIF-1α induces both pro-apoptotic (BNIP3, NOXA, NIX, RTP801) and anti-apoptotic (FOXO3, IAP-2 and survivin) genes (19-23). For each cellular phenotype, the death or survival induced by hypoxia seems to depend on degree. As used in this study, 1% O_2 hypoxia did not promote cell death in pancreatic cancer cells by itself, while others have shown that severe hypoxia (0.1% O_2) or anoxia induced cell death (24). In addition, 1% O_2 hypoxia induces both pro- and anti-apoptotic genes; the total balance, however, seems to be anti-apoptotic dominant (Fig. 5). One of the reasons for the negative contribution of the physiological level of ASC to cellular death under 1% O_2 hypoxia might be a loss of Bax. As a result, the role of ASC under hypoxia remains unclear. It is also unknown whether ASC is a direct target gene of transcriptional factor HIF-1α. ASC might have a greater capacity for cellular death under severely hypoxic conditions, thus further investigation is required to clarify its precise role under hypoxia.

To overcome this anti-apoptotic-dominant condition under hypoxia, we infected adenovirus-expressing ASC into pancreatic cancer cells. ASC gene induction successfully promoted cell death in pancreatic cancer cells even under normoxia, and was enhanced by 1% O_2 hypoxia (Figs. 4 and 5). There are two ASC-associated death signaling pathways; namely, the p53-dependent Bax-mitochondrial pathway and the p53-independent death receptor-caspase 8 pathway (10). The latter seems to be the main pathway of ASC-mediated cell death under hypoxia, since hypoxia causes Bax repression (Fig. 3). ASC has 2 protein adaptor domains, and therefore has the potential to bind to various types of molecules, regulating cellular death in response to various death stimuli (9-12). Since there have been no reports describing the relationship between ASC and hypoxia, subsequent study should identify the hypoxia-inducible genes which collaborate with ASC under hypoxia. In any event, the findings of the current study suggest that ASC gene induction is a potential new treatment strategy for pancreatic cancer.

Acknowledgements

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