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Ultraviolet enhances the sensitivity of pancreatic cancer cells to gemcitabine by activation of 5′ AMP-activated protein kinase

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ABSTRACT

Although gemcitabine is recognized as the standard drug for the treatment of advanced pancreatic cancer, the clinical outcome is not satisfactory. We recently reported that relatively high dose ultraviolet-C (UV-C; 200 J) inhibits cell growth by desensitization of epidermal growth factor receptor (EGFR) in human pancreatic cancer cells. In the present study, we investigated the combination effects of low dose UV-C (10 J) and gemcitabine on apoptosis and cell growth in these cells. UV-C enhanced gemcitabine-induced suppression of cell viability. In addition, the combination use clearly induced apoptosis, while neither UV-C nor gemcitabine alone did. Concurrently, combination use caused the decrease in the EGFR protein level and reduced EGF-induced activation of Akt pathway, subsequently resulting in accumulation of β-catenin. The order of the treatment with UV-C and gemcitabine did not affect their synergistic effects on apoptosis and cell growth. Interestingly, combination use synergistically induced phosphorylation of 5′ AMP-activated protein kinase (AMPK) alpha at Thr172 and acetyl-CoA carboxylase at Ser79 as a downstream molecular target of AMPK. AMPK activator, 5-aminoimidazole-4-carboxamide-1-β-riboside, induced apoptosis and suppressed cell growth in these cells, thus suggesting that combination effects of UV-C and gemcitabine is due to the activation of AMPK. Together, our findings could provide a new aspect of pancreatic cancer therapy.

1. Introduction

Because of the difficulty in early diagnose of pancreatic cancer, most patients with this malignancy have already reached an advanced stage when the first symptoms appear. The standard treatment for advanced pancreatic cancer is chemotherapy. Gemcitabine is a nucleoside analogue of deoxycytidine that is enzymatically activated inside the cell where it subsequently inhibits DNA synthesis and induces apoptosis [1] and has been the first line drug for pancreatic cancer. However, the median survival of patients treated with gemcitabine is not satisfactory. Moreover, a number of studies have compared gemcitabine alone with gemcitabine-based combinations, such as fluorouracil, capecitabine, cisplatin, docetaxel, irinotecan, oxaliplatin, or pemetrexed, but they added no clear survival benefit [2]. Therefore, the breakthrough development of treatments for unresectable pancreatic cancer is required urgently.

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases [3]. Binding of ligands such as epidermal growth factor (EGF) to the EGFR leads to receptor dimerization and autophosphorylation [4]. The autophosphorylation of the EGFR at tyrosine residues activates downstream signalling, including Akt-glycogen synthase kinase (GSK)-3β pathway, thus resulting in the stimulation of cell proliferation [5]. The EGFR has been reported to be overexpressed in pancreatic cancer [6]. We have recently reported that the blockade of EGF stimulation significantly suppressed pancreatic cancer cell growth, suggesting that the EGFR pathway plays an important role in proliferation of these cells [7]. Therefore, EGFR-mediated pathways appear to be important potential targets for new therapies for this malignancy. Moreover, the addition of EGFR-targeted therapy to gemcitabine in advanced pancreatic cancer has been demonstrated to provide a small, but statistically significant, survival benefit [8].

Ultraviolet (UV) radiation from sunlight is sorted by wavelength regions: long-wavelength UV-A (320–400 nm), medium-wavelength UV-B (280–320 nm) and short-wavelength UV-C (200–280 nm). In general, UV-A and UV-B are recognized as the major carcinogenic components of sunlight [9], and UV-C is used for studying DNA damage and cellular DNA repair process, and commonly applied for equipments such as water sterilization. We recently reported that UV-C irradiation suppresses cell growth via downregulation of EGFR in human pancreatic cancer cells [10].

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Moreover, our recent study shows that UV-C can induce evasion of colon cancer cells from oncogenic stimulation of EGF [11]. Hence, UV-C might be applied for clinical strategy against human cancers.

5′ AMP-activated protein kinase (AMPK) is a central cellular energy sensor which may be a crucial factor in the interaction between metabolism and cancer [12]. AMPK activation results in the restoration of energy levels through regulation of metabolism and growth [13], and the loss of AMPK activity causes cell proliferation, suggesting that AMPK is a potential target for anti-cancer therapy. In this study, we tried the combination use of low dose UV-C and gemcitabine in pancreatic cancer cells and found that this exerts synergistic effects on the induction of apoptosis and suppression of cell growth via activation of AMPK.

2. Materials and methods

2.1. Materials

Gemcitabine was obtained from Eli Lilly Co. (Indianapolis, IN, USA) and 5-aminoimidazole-4-carboxamide-1-β-riboside (AICAR) was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). EGF was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against EGFR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other antibodies were purchased from Cell Signalling (Beverly, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) cell proliferation kit I and Cell Proliferation ELISA (BrdU) were obtained from Roche Diagnostics Co. (Indianapolis, IN, USA).

2.2. Cell culture

Panc1 and KP3 pancreatic cancer cells were provided from American Type Culture Collection (Manassas, VA, USA). They were grown in Roswell Park Memorial Institute (RPMI) 1640 (Invitrogen, San Diego, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 incubator at 37 °C.

2.3. UV-C exposure

UV-C exposure to cells was performed in UVC 500 UV Crosslinker (GE Healthcare) with 0–500 J/m^2 (J) UV at 254 nm. After aspiration of the growth medium, the cells were exposed to UV-C (0 or 10 J) and then incubated in the growth medium for the indicated periods.

2.4. Cell viability assay and BrdU incorporation assay

In cell viability assay, the cells (5×10^3/well) were seeded onto 96-well plates and 24 h later, the cells were exposed to 10 J of UV-C and/or gemcitabine at the indicated concentrations. These cells were then incubated in RPMI for 48 h and the remaining cells were finally counted by MTT cell proliferation kit. For the latter assay, the cells (7×10^3/well) were seeded onto 96-well plates and 48 h later, the cells were exposed to 10 J of UV-C and/or 10 μM of gemcitabine or 10 mM of AICAR. These cells were then incubated in RPMI without FCS for 48 h. BrdU incorporation was finally measured using Cell Proliferation ELISA. All assays were done at least in triplicate.

2.5. Western blot analysis

The cells were lysed in lysis buffer and were examined by Western blot analysis as previously described [14].

2.6. Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously [15]. The cells were first exposed to UV-C (10 J) and incubated for 24 h at 37 °C, followed by exposure to Hoechst 33258 (Wako, Tokyo, Japan) for 30 min. The cells were then examined by fluorescence microscopy, BIOREVO (BZ-9000) (Keyence, Tokyo, Japan).

2.7. Image analysis

The protein band intensities in the Western blot analysis were determined by integrating the optical density over the band area (band volume) using the NIH image software program (Image J ver. 1.32). Based on the intensity of the control protein band on the X-ray film, the protein samples were quantitatively compared.

3. Results

UV-C enhanced gemcitabine-induced cytotoxicity in Panc1 and KP3 pancreatic cancer cells

As shown in Fig. 1A, 10 μM of gemcitabine alone for 48 h caused approximately 20% reduction in cell viability and only 30% reduction was seen even when the cells were treated with 100 μM of gemcitabine alone in KP3 cells (Fig. 1A). However, pretreatment with UV-C significantly increased gemcitabine-induced cytotoxicity in KP3 cells, while 10 J of UV-C alone did not affect KP3 cell viability (Fig. 1A). Ten μM and 30 μM of gemcitabine combined with UV-C caused 50% and 75% reduction in cell viability in KP3 cells, respectively. As well, we used another pancreatic cancer cell line, Panc1, and observed similar effect to that in KP3 cells (Fig. 1B). These results suggest that UV-C enhances gemcitabine-induced suppression of cell viability in pancreatic cancer cells.

3.1. Combination use of UV-C and gemcitabine induced apoptosis in pancreatic cancer cells

In order to elucidate how UV-C enhances gemcitabine-induced suppression of cell viability, we next examined the effects of UV-C and gemcitabine on cell apoptosis in KP3 cells. Apoptotic cells are easily detected by the Hoechst 33258 staining. In addition, PARP helps cells to maintain their viability, while cleavage of PARP induces apoptosis. In Fig. 1C, while 10 J of UV-C alone as well as 10 μM of gemcitabine alone slightly increased the number of Hoechst 33258-positive cells in KP3 cells, combination use caused a marked increase in the number of those cells (Fig. 1C). Similarly, while either UV-C or gemcitabine alone did not induce PARP cleavage, this was clearly observed when the cells were treated with both UV-C and gemcitabine (Fig. 1D). These results suggest that synergistic effect of UV-C and gemcitabine on the suppression of cell viability is, at least in part, due to the induction of cell apoptosis.

3.2. UV-C enhanced gemcitabine-induced cell growth suppression in pancreatic cancer cells

As depicted in Fig. 2A, UV-C had little effect on BrdU incorporation and 10 μM of gemcitabine alone caused a slight suppression of its incorporation (Fig. 2A). As expected, when the cells were exposed to UV-C and then treated with gemcitabine for 48 h, BrdU incorporation was significantly suppressed ($p = 0.0047$), compared with gemcitabine-treated cells (Fig. 3A). We recently reported that the EGFR pathway plays an important role in pancreatic cancer cell proliferation [7]. Therefore, we next examined the effect of UV-C and gemcitabine on the protein level of EGFR in KP3 cells. In Fig. 2B, whereas either UV-C or gemcitabine had little effect on

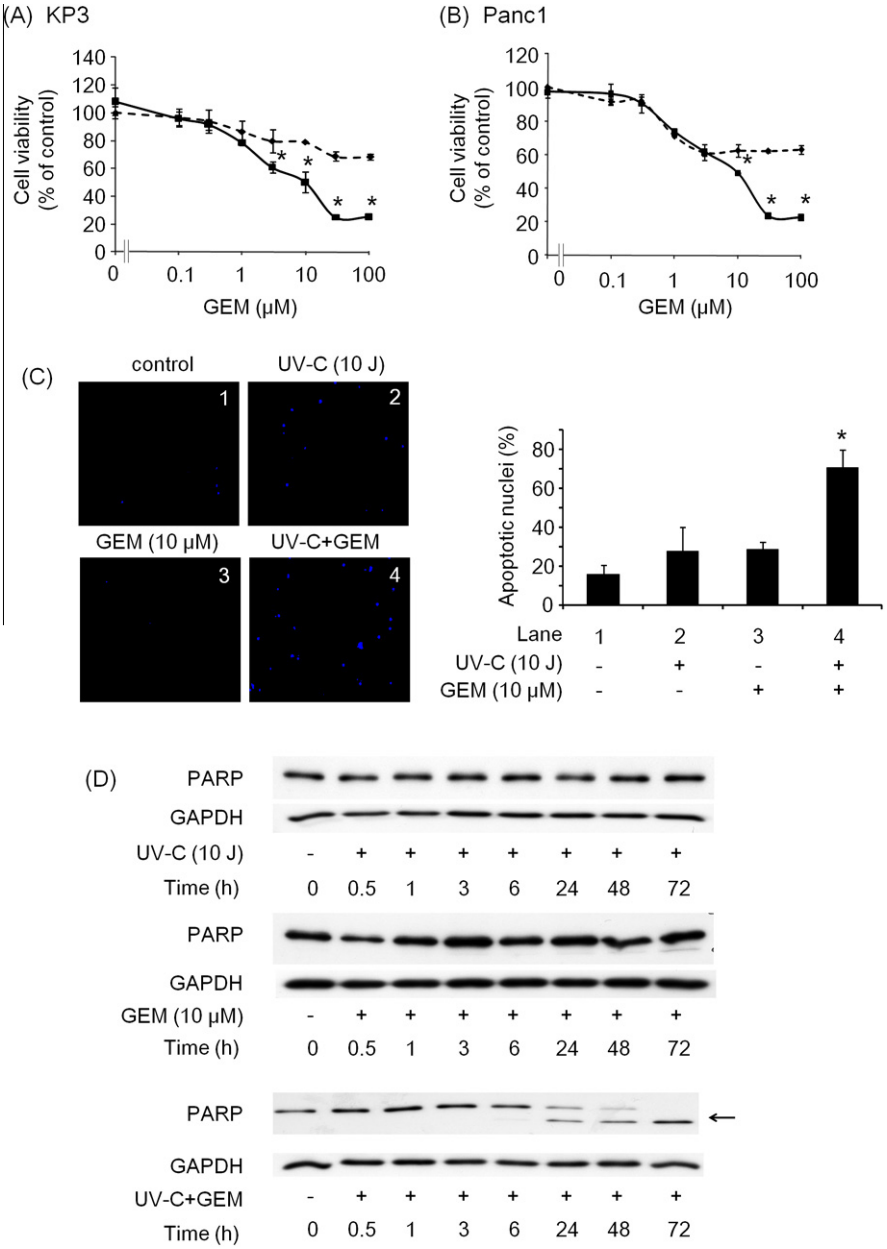

Fig. 1. (A and B) UV-C enhanced the inhibition of cell growth by gemcitabine in pancreatic cancer cells. The KP3 (A) and Panc1 (B) cells were first exposed to UV-C (0 or 10 J) and then incubated with gemcitabine (GEM; 0–100 μM) for 48 h. Cell viability assay was then performed using the MTT cell proliferation kit I. Results are expressed as percentage of growth with 100% representing untreated control cells. Bars designate SD of triplicate assays. Broken line: GEM alone; solid line: UV-C + GEM. (C) The combination use of UV-C and GEM induced DNA fragmentation in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for 48 h. They were then exposed to Hoechst 33258 (blue signal) and were examined by fluorescence microscopy. The numbers of Hoechst 33258-positive cells (apoptotic nuclei) from five randomly chosen fields (×40) were counted, respectively. Bars designate SD of triplicate assays. The asterisk indicates significant difference ($p < 0.05$), as compared with the cells treated with GEM alone (lane 3). (D) The combination use of UV-C and GEM induced PARP cleavage in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without gemcitabine 10 μM of GEM for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against PARP and GAPDH. The arrow indicates cleaved PARP.

the EGFR protein level, combination use markedly decreased EGFR level (Fig. 2B). Therefore, it seems that cell growth suppression by combination use of UV-C and gemcitabine is due to the decrease in EGFR protein level.

3.3. Combination use of UV-C and gemcitabine suppressed EGF-induced activation of Akt-GSK-3β pathway in pancreatic cancer cells

Through EGF-binding to cell surface EGFR, it activates an extensive network of signal transduction pathways including the Akt pathways, which regulates multiple biological processes including survival, proliferation, and cell growth [16]. Therefore, we next examined the effect of UV-C and gemcitabine on EGF-induced phosphorylation of Akt in KP3 cells. EGF induced phosphorylation of Akt within 5 min and this was decreased thereafter (Fig. 2C). UV-C and gemcitabine alone slightly suppressed EGF-induced phosphorylation of Akt (Fig. 2C). Moreover, the combination use markedly suppressed EGF-induced phosphorylation of Akt (Fig. 3C). GSK-3β is a critical downstream element of the Akt pathway, and its activity can be inhibited by Akt-mediated phosphory-

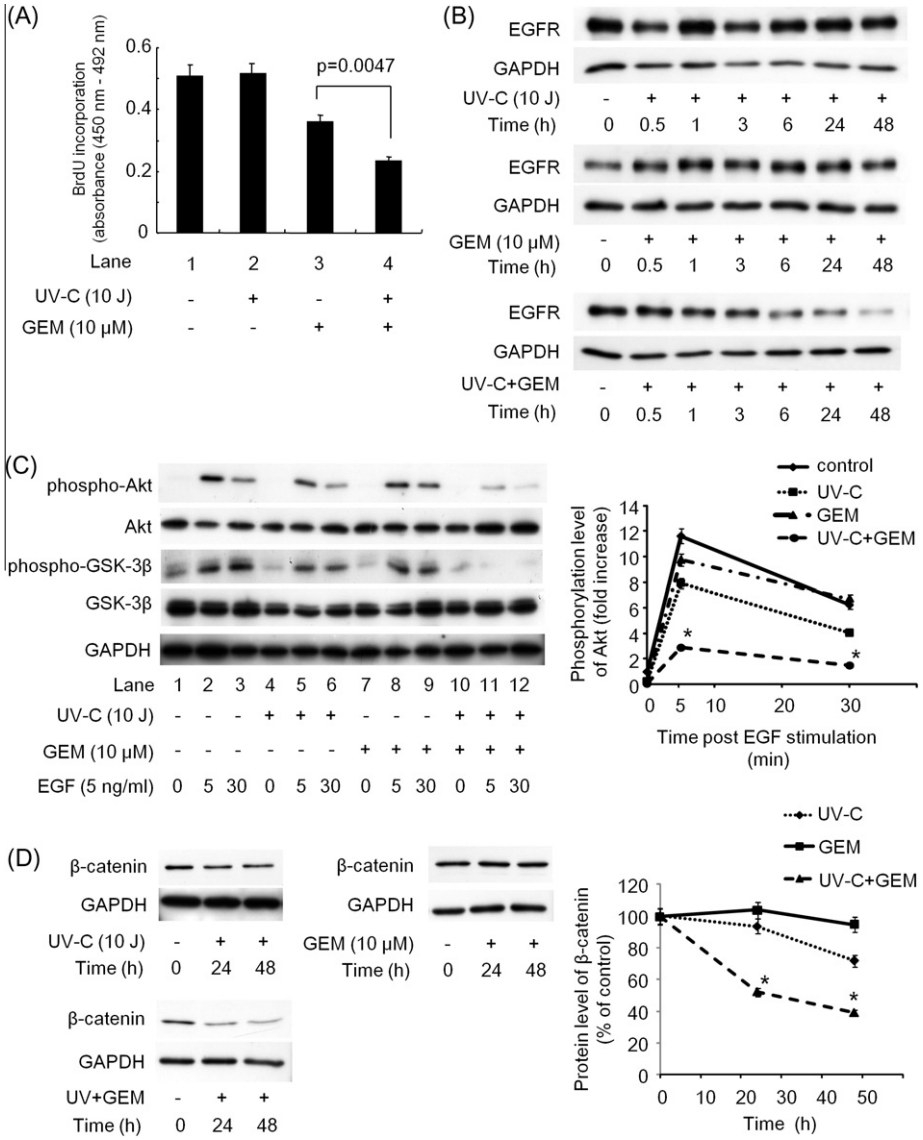

Fig. 2. (A) The combination use of UV-C and gemcitabine (GEM) inhibited BrdU incorporation in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for 48 h and the measurement of BrdU incorporation during DNA synthesis were performed using cell proliferation ELISA (BrdU). Results are expressed as the absorbance (OD 405 nm–492 nm). Bars designate SD of triplicate assays. The asterisk indicates significant difference ($p = 0.0047$), between the indicated pairs. (B) The combination use of UV-C and GEM caused the decrease in the EGFR protein level in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against EGFR and GAPDH. (C) The combination use of UV-C and GEM suppressed EGF-induced Akt pathway in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for 24 h. They were then stimulated with EGF (5 ng/ml) for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against phospho-Akt, Akt, phospho-GSK3β, GSK3β and GAPDH. Right line graph shows the quantification data for the relative phosphorylation levels of Akt, after normalization with respect to total Akt, as determined by densitometry. Bars designate SD of triplicate assays. $^{*}p < 0.05$: compared to the cells treated with GEM alone. (D) The combination use of UV-C and GEM caused the decrease in the β-catenin protein level in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against β-catenin and GAPDH. Right line graph shows the quantification data for the relative levels of β-catenin, after normalization with respect to GAPDH, as determined by densitometry. Bars designate SD of triplicate assays. $^{*}p < 0.05$: compared to the cells treated with GEM alone.

lation of GSK-3β at Ser9 [17]. As expected, EGF failed to phosphorylate GSK-3β at Ser9 in the cells treated with UV-C and gemcitabine (Fig. 3C).

Deregulation of Wnt-β-catenin cascades has been reported in many types of cancers, including pancreatic cancer [18] and phosphorylated form of GSK-3β caused the accumulation of β-catenin. As shown in Fig. 2D, combination use of UV-C and gemcitabine caused the decrease in the protein level of β-catenin, whereas either UV-C or gemcitabine alone failed to affect. Together, our results suggest that combination use of UV-C and gemcitabine induces the suppressive effect on cell growth, at least in part, by inhibiting the Akt pathway.

3.4. The order of the treatment with UV-C and gemcitabine did not affect their synergistic effects on apoptosis and cell growth

We next examined which is more effective treatment to enhance the sensitivity to gemcitabine, pre- or post-treatment with UV-C. Either gemcitabine or UV-C did not affect the protein level of EGFR as well as cleavage of PARP (Fig. 3A). When the cells were

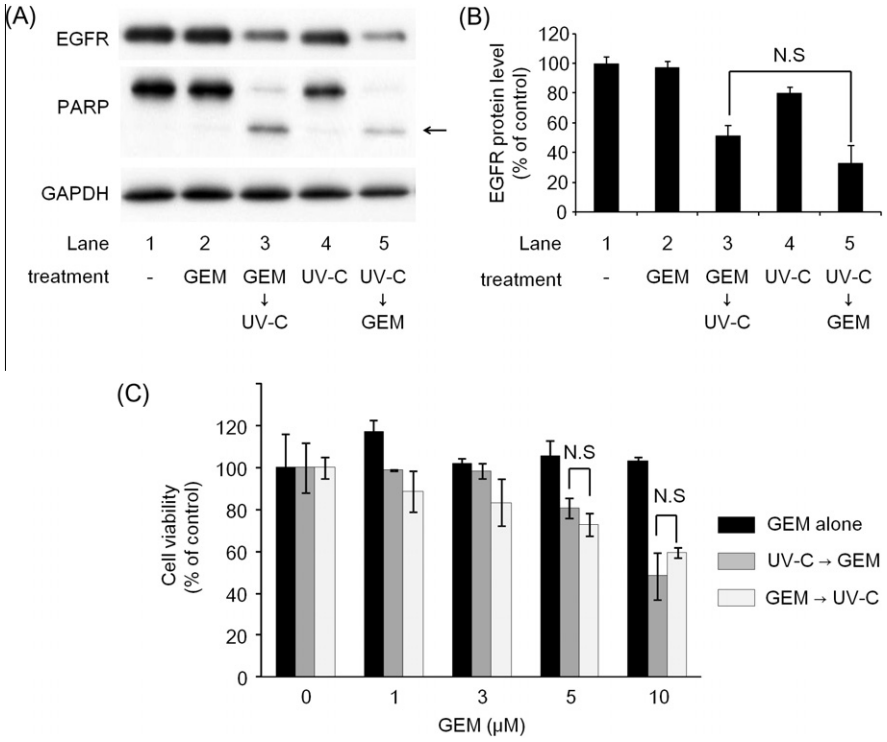

Fig. 3. The order of treatment with UV-C and gemcitabine (GEM) is not critical for their combination effect in KP3 cells. (A) The cells (lanes 4 and 5) were exposed to 10 J of UV-C and then treated with (lane 5) or without (lane 4) GEM (10 μM) for 8 h. The media were then exchanged to the fresh one and incubated for another 40 h. The other cells were first treated with (lanes 2 and 3) or without (lane 1) GEM (10 μM) for 8 h and then exposed to 10 J of UV-C (lane 3) or not (lanes 1 and 2), and subsequent incubation for another 40 h in the fresh media. Protein extracts were then harvested and examined by Western blotting using antibodies against EGFR, PARP and GAPDH. The arrow indicates cleaved PARP. (B) The bar graph shows the quantification data for the relative levels of EGFR after normalization with respect to GAPDH, as determined by densitometry. Bars designate SD of triplicate assays. No significant difference exists between the indicated pairs. (C) Some cells (grey bar) were first exposed to 10 J of UV-C and then treated with GEM at the indicated concentrations for 8 h. The media were then exchanged to the fresh one and incubated for another 40 h. The other cells (black and white bars) were first treated with GEM at the indicated concentrations for 8 h and then exposed to 10 J of UV-C (white bar) or not (black bar), and subsequent incubation for another 40 h in the fresh media. Cell viability assay was then performed using the MTT cell proliferation kit I. Results are expressed as percentage of growth with 100% representing untreated control cells. Bars designate SD of triplicate assays.

pretreated with gemcitabine for 8 h and then exposed to UV-C (gemcitabine → UV-C treatment), we observed the decrease in EGFR protein level in addition to the induction of PARP cleavage, which are similar to the results obtained from UV-C → gemcitabine treatment (Fig. 3A and B). Moreover, similar results were observed in cell viability assay (Fig. 3C). Taken together, our findings strongly suggest that the order is not important for combination use of UV-C and gemcitabine.

3.5. The combination use of UV-C and gemcitabine induced activation of AMPKα in pancreatic cancer cells

In order to investigate the mechanism underlying combination effects of UV-C and gemcitabine, we next examined several kinase cascades including AMPK. Interestingly, whereas either UV-C or gemcitabine had little effect on phosphorylation of AMPKα (Thr172) and acetyl-CoA carboxylase (ACC; Ser 79) as a downstream molecular target of AMPK, the combination use caused a marked phosphorylation of both proteins (Fig. 4A). These results led us to speculate that activation of AMPK induced by UV-C and gemcitabine exert anti-cancer effect, such as the induction of apoptosis and the suppression of cell growth.

To verify these findings, we used AMPK activator, AICAR and found that increasing doses of AICAR caused cleavage of PARP (Fig. 4B) and this increased Hoechst 33258-positive cells (Fig. 4C), suggesting that activation of AMPK leads to apoptosis in pancreatic cancer cells. Moreover, since AICAR suppressed BrdU incorporation (Fig. 4D), indicating that activation of AMPK inhibits cell cycle. Taken together with the results shown above, it is most likely that the synergistic effects of UV-C and gemcitabine are exerted through the activation of AMPK.

4. Discussion

We have recently reported the availability of UV-C for the treatment of human pancreatic cancers [10]. In that article, we used relatively high dose UV-C (200 J), and showed that UV-C has a suppressive effect on cell proliferation in pancreatic cancer cells, but not normal pancreas epithelial cells. In the present study, we demonstrated the synergistic effects of low dose UV-C (10 J) and gemcitabine. First, we showed that UV-C enhanced gemcitabine-induced cytotoxicity in Panc1 and KP3 cells (Fig. 1A and B). This combination use caused cell apoptosis (Fig. 1C and D) as well as inhibition of cell proliferation (Fig. 2A), concurrent with downregulation of EGFR which exerts oncogenic signalling, such as the Akt pathway (Fig. 2B–D). These results indicate that the synergistic effect against pancreatic cancer cells on cell viability is due to the induction of cell apoptosis and suppression of cell proliferation.

Gemcitabine is now regarded as the first-line agent for advanced pancreatic cancer, but the median survival time of patients treated with gemcitabine is not satisfactory. Importantly, it has been reported that gemcitabine induces cell apoptosis by activating p38 mitogen-activated protein kinase (MAPK) in PK-1 and PCI-43 human pancreatic cancer cell lines [19]. However, in this study, the combination use of UV-C and gemcitabine did not induce phosphorylation of p38 MAPK in Panc1 and KP3 cells (data not shown). Therefore, it is likely that the synergistic effect of UV-C and gemcitabine is independent of the p38 MAPK pathway.

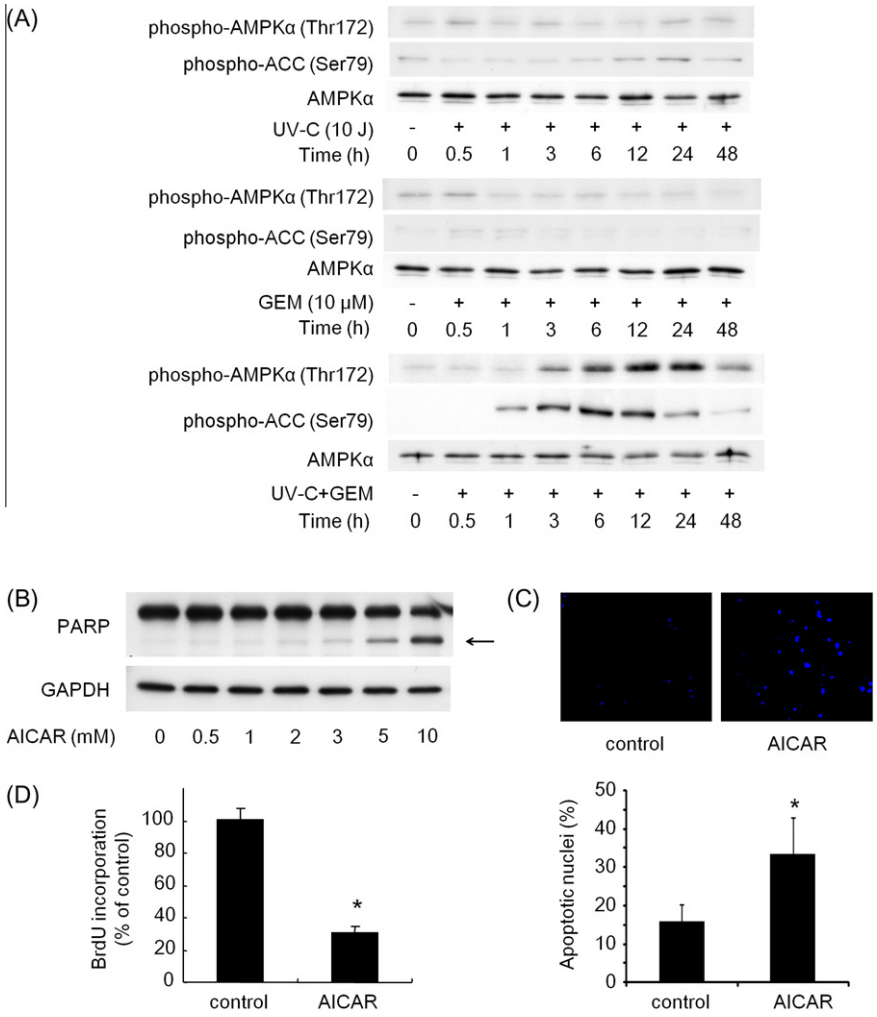

Fig. 4. (A) The combination use of UV-C and gemcitabine (GEM) caused the activation of AMPK in KP3 cells. The KP3 cells were first exposed to UV-C (0 or 10 J) and then incubated with or without 10 μM of GEM for the indicated periods. Protein extracts were then harvested and examined by Western blotting using antibodies against phospho-AMPKα (Thr172), phospho-acetyl-CoA carboxylase (ACC; Ser79) and AMPKα. (B) AMPK activator, AICAR, induced apoptosis in KP3 cells. The KP3 cells were treated with AICAR at the indicated concentrations for 24 h. Protein extracts were then harvested and examined by Western blotting using antibodies against PARP and GAPDH. The arrow indicates cleaved PARP. (C) The KP3 cells were treated with 10 mM of AICAR for 24 h and were then exposed to Hoechst 33258 (blue signal) and were examined by fluorescence microscopy. The numbers of Hoechst 33258-positive cells (apoptotic nuclei) from five randomly chosen fields (×40) were counted, respectively. Bars designate SD of triplicate assays. The asterisk indicates significant difference ($p < 0.05$), as compared with the control cells. (D) The KP3 cells were treated with 10 mM of AICAR for 24 h and the measurement of BrdU incorporation during DNA synthesis were performed using cell proliferation ELISA (BrdU). Results are expressed as the absorbance (OD 405 nm–492 nm). Bars designate SD of triplicate assays. The asterisk indicates significant difference ($p < 0.05$), as compared with the control cells.

AMPK is a serine/threonine protein kinase, which serves as an energy sensor in eukaryotic cell types. Increasing evidence shows that AMPK activation strongly suppresses cell proliferation in normal cells as well as in cancer cells, indicating that AMPK functions as a suppressor of cell proliferation by controlling a variety of cellular events in these cells [20]. Metformin, which is widely used as an anti-diabetic drug, has been recently reported to be associated with a reduced risk of cancer associated with insulin resistance [12,21]. The effects of metformin are explained by the activation of AMPK, which regulates cellular energy metabolism [22]. Of interest, in the present study, the combination use of UV-C and gemcitabine induced activation of AMPK (Fig. 4A). The order of the treatment with UV-C and gemcitabine is not critical for their combination effect (Fig. 3). Moreover, we observed the activation of AMPK in the cells, which were first treated with gemcitabine for 8 h and then exposed to UV-C, subsequent incubation for another 40 h (data not shown), consistently with the results shown in Fig. 3. However, further investigation is necessary to elucidate how the combination use of UV-C and gemcitabine activates AMPK.

In summary, we presented that UV-C enhances the sensitivity to gemcitabine in pancreatic cancer cells via activation of AMPK. Our novel findings could provide a fresh development for human pancreatic cancer therapy, although the development of devices that supply UV-C efficiently, for example with endoscopic approach, is also required for future clinical application.

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