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EPA, an Omega-3 Fatty Acid, Induces Apoptosis in Human Pancreatic Cancer Cells: Role of ROS Accumulation, Caspase-8 Activation, and Autophagy Induction

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ABSTRACT

In a recent study, we showed that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two common omega-3 fatty acids, can cause ROS accumulation and subsequently induce caspase-8-dependent apoptosis in human breast cancer cells (Kang et al. [2010], PLoS ONE 5: e10296). In this study, we showed that the pancreas has a unique ability to accumulate EPA at a level markedly higher than several other tissues analyzed. Based on this finding, we sought to further investigate the anticancer actions of EPA and its analog DHA in human pancreatic cancer cells using both in vitro and in vivo models. EPA and DHA were found to induce ROS accumulation and caspase-8-dependent cell death in human pancreatic cancer cells (MIA-PaCa-2 and Capan-2) in vitro. Feeding animals with a diet supplemented with 5% fish oil, which contains high levels of EPA and DHA, also strongly suppresses the growth of MIA-PaCa-2 human pancreatic cancer xenografts in athymic nude mice, by inducing oxidative stress and cell death. In addition, we showed that EPA can concomitantly induce autophagy in these cancer cells, and the induction of autophagy diminishes its ability to induce apoptotic cell death. It is therefore suggested that combination of EPA with an autophagy inhibitor may be a useful strategy in increasing the therapeutic effectiveness in pancreatic cancer. J. Cell. Biochem. 114: 192–203, 2013.

KEY WORDS: EICOSAPENTAENOIC ACID; DOCOSAHEXAENOIC ACID; OMEGA-3 POLYUNSATURATED FATTY ACIDS; PANCREATIC CANCER; APOPTOSIS; AUTOPHAGY

In the United States, pancreatic cancer is the fourth leading cause of cancer mortality in both men and women [Jemal et al., 2008]. The 5-year survival rate of patients with pancreatic cancer is particularly low (below 5%) because this cancer often only produces symptoms when it has already metastasized and presently there are specific tools for its early detection. Complete surgical resection remains the only curative treatment. Non-surgical treatment options such as chemotherapy or radiotherapy are not very effective. Therefore, novel preventive and therapeutic strategies are needed for reducing the incidence of pancreatic cancer and also for improving its therapeutic outcomes.

The use of certain bioactive dietary agents is considered an attractive approach for the prevention and treatment of certain types of cancers. Epidemiological as well as experimental studies have shown that diet rich in fruits, vegetables, and fish oil are beneficial for the protection against certain human malignancies including pancreatic cancer [Shirota et al., 2005; Vainio and Weiderpass, 2006; Dekoj et al., 2007; Hering et al., 2007; Funahashi et al., 2008].

Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.Masayuki Fukui and Ki Sung Kang contributed almost equally to the work described in this study.

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Docosahexaenoic acid (DHA)[4] and eicosapentaenoic acid (EPA)[5], two well-known omega-3 fatty acids (FAs), have been reported to have anticancer activity in many studies [Shirota et al., 2005; Lim et al., 2009; Kang et al., 2010]. A number of mechanisms have been proposed for their anticancer actions, including suppression of neoplastic transformation, inhibition of cell cycle, enhancement of apoptosis, and antiangiogenicity [Bartram et al., 1993; Shirota et al., 2005; Dekoj et al., 2007]. Studies have shown that accumulation of certain polyunsaturated fatty acids (PUFAs) would lead to increased lipid peroxidation and increased formation of lipid hydroperoxides and other lipid degradation products that may be deleterious to cancer cells [Welsch, 1995; Koh et al., 1997; Cognault et al., 2000; Gago-Dominguez et al., 2005]. Moreover, it has been shown that the presence of antioxidants abrogates their anticancer activity in cultured human breast cancer cells [Kang et al., 2010], whereas the presence of prooxidants enhances their anticancer activity [Chajes et al., 1995; Colas et al., 2005], thereby suggesting the important role of ROS in mediating their anticancer actions.

Apoptosis is an important mechanism of cell death [Evan and Vousden, 2001]. Apoptosis can usually be activated by two major apoptotic pathways, namely, the intrinsic mitochondrial cell death pathway and the extrinsic death receptor-mediated cell death pathway [Shelton et al., 2010]. The intrinsic pathway leads to disruption of mitochondrial membranes, release of the mitochondrial cytochrome c into cytoplasmic compartment, which then binds to the apoptotic protease-activating factor (Apaf) complex and triggers the activation of caspases [Mellier et al., 2010]. In contrast, the extrinsic cell death pathway is initiated by the selective activation of the death receptors by ligands like the tumor necrosis factors [Ashkenazi and Dixit, 1998; Ashkenazi, 2002; Deng et al., 2002; Le Blanc et al., 2008]. In Type I cells, activation of the death receptors will recruit and activate the apoptosis-initiating proteases caspase-8/10 through the Fas-associated death domain (FADD). Caspase-8/10, in turn, activates the executioner caspases (e.g., caspase-3, -6, and -7), which execute apoptotic cell death. However, in Type II cells (such as pancreatic cancer cells), caspase-8/10 engages the mitochondrial apoptotic pathway via activating the pro-apoptotic Bcl-2 family member Bid, which then interacts with two other pro-apoptotic family members, Bax and Bak, to induce the release of cytochrome c and Smac/Diablo from mitochondria. Cytochrome c, together with Apaf-1, activates caspase 9, which contributes to further activation of caspase-3, -6, and -7. Smac/Diablo binds to inhibitor of apoptosis proteins (IAPs), preventing the IAPs from physically binding caspase-3, hence promoting further caspase-3 activation.

Autophagy is a catabolic process that targets cellular organelles (include damaged mitochondria) and cytoplasmic constituents to lysosomes for degradation [Mizushima et al., 2008]. There is increasing evidence showing that autophagy has complex and paradoxical roles in tumorigenesis, tumor progression, and cancer chemotherapy [Liu and Ryan, 2012]. While autophagy can function to promote tumor cell survival [Degenhardt et al., 2006], it can also induce cancer cell death [Mathew et al., 2007; Rosenfeldt and Ryan, 2011]. It is, therefore, important to understand whether autophagy promotes survival or enhances cancer cell death under certain conditions.

In this study, we sought to investigate the anticancer actions of EPA in human pancreatic cancer cells both in vitro and in vivo (as cancer cell xenografts in athymic nude mice), with a focus on assessing the role of oxidative stress, apoptosis, and autophagy in the mechanism of their anticancer actions. A better understanding of the mechanism of the anticancer actions of these omega-3 FAs will aid in the development of effective cancer chemotherapeutic strategies involving their use as potential anticancer adjuvants. This study was partly prompted by our observation showing that among various tissues measured, very high levels of EPA are present in the pancreases of mice following oral administration of a fish oil-supplemented diet.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

DHA, EPA, linoleic acid (18:2n − 6), arachidonic acid (20:4n − 6), boron trifluoride, and fetal bovine serum (FBS) were obtained from Sigma Chemical, Co. (St. Louis, MO). Dubecco's modified Eagle's medium (DMEM) and an antibiotics solution containing 10,000 U/mL penicillin and 10 mg/mL streptomycin were obtained from Invitrogen (Carlsbad, CA). The McCoy's 5A medium and trypsin–EDTA mixture (containing 0.25% trypsin and 0.02% EDTA) were obtained from Lonza Walkersville (Walkersville, MD). Caspase inhibitors (zVAD-FMK, IETD-FMK, LEHD-FMK, and zVEID-FMK) were obtained from EMD Chemicals (Gibbstown, NJ). Scramble siRNAs and caspase 8-specific siRNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents used in this study were obtained from standard suppliers and were of analytical grade or higher.

CELL CULTURE AND MTT ASSAY

The MIA-PaCa-2 and Capan-2 human pancreatic cancer cells were obtained from the American Type Culture Collection (Rockville, MD). MIA-PaCa-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), and Capan-2 cells were maintained in McCoy's 5A medium supplemented with 10% FBS. These cells were incubated at 37°C under 5% CO_2. Cells were subcultured every 3–4 days. The cells were seeded in 96-well plates at a density of 5,000 cells per well. The 10 mM stock solution of DHA or EPA (dissolved in 200-proof ethanol) was diluted in the culture medium immediately before addition to each well at the desired final concentration(s), and the treatment usually lasted for 72 h.

For the initial testing of the cell viability change, the MTT assay was used. MTT (at 5 mg/mL) was added to each well at a final concentration of 500 μg/mL. After the mixture in each well was incubated for 1 h, it was removed and DMSO (100 μL) was added, and the absorbance was read with a UV max microplate reader (Molecular Device, Palo Alto, CA) at 560 nm. The relative cell viability was expressed as a percentage of the control well that was treated with the vehicle only.

WESTERN BLOTTING

For Western blotting, cells were washed first, and then were suspended in 100 μL lysis buffer (containing 20 mM Tris–HCl,

150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (v/v), 10 mM NaF, 2 mM Na_3VO_4, and a protease inhibitor cocktail, pH 7.5). The amount of proteins was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). After an equal amount of proteins was loaded in each lane, they were separated by 10% (w/v) SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then electrically transferred to a polyvinylidene difluoride membrane (Bio-Rad). After blocking the membrane with 5% (w/v) skim milk, target proteins were immunodetected using specific antibodies. All primary antibodies for Western blotting were obtained from Cell Signaling Technology (Beverly, MA) and used at 1:1,000 dilution. Thereafter, the horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Invitrogen) was applied as the secondary antibody, and the positive bands were detected using the Amersham ECL Plus Western blotting detection reagents (GE Health care, Piscataway, NJ).

MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species (ROS) were detected using the 2′,7′-dichlorofluorescin diacetate (H_2-DCF-DA) method. Cells were first cultured in 96-well plate and treated with 100 μM omega-3 FAs for 8 h, and then 10 μM H_2-DCF-DA was added to each well. After incubation for 10 min at 37°C, the liquid was removed and PBS was added. Intracellular ROS accumulation was observed and photographed under a fluorescence microscope (AXIO; Carl Zeiss Corporation, Germany).

IMMUNOCYTOCHEMICAL ANALYSIS

Immunofluorescence staining was done in cells cultured in chamber slides. Briefly, after EPA treatment, cells were fixed in 2% formaldehyde and then incubated in ice-cold 100% methanol. Then, cells were blocked with 5% normal goat serum, incubated with rabbit anti-LC3B antibody (Cell Signaling) followed by incubation with the FITC-conjugated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA). The nuclei were counter-stained with Hoechst-33342. The slides were examined under a fluorescence microscope.

TRANSFECTION OF siRNAs

To study the role of Beclin 1 in EPA-induced pancreatic cancer cell death, Beclin 1 siRNAs (Santa Cruz) were used to selectively knock down its expression in MIA-PaCa-2 cells. The cells were seeded for 24 h and reached a density of 30–50% confluence at the time of transfection. Then, the Beclin1 siRNAs or the negative control siRNAs were used for transfection with Lipofectamine 2000 according to the manufacturer's instructions. The transfected cells were maintained in culture for 24 h before harvest and further analyses. The efficacy of the siRNA knockdown of the target protein expression was determined by Western blot analysis with specific antibodies.

ANALYSIS OF CELLULAR MORPHOLOGICAL CHANGES USING TRANSMISSION ELECTRON MICROSCOPY

Cells were harvested using trypsin–EDTA and fixed in 2% glutaraldehyde for 4 h, and centrifuged to form pellets. Sample preparation was carried out according to a published method [Hanaichi et al., 1986]. Briefly, the pellets were rinsed in 0.1 M cacodylate buffer (purchased from Electron Microscopy Sciences, Hatfield, PA) and post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences). Cell pellets were dehydrated through a graded series of ethanol and then passed through a propylene oxide twice. They were placed in propylene oxide/Embed 812 resin (Electron Microscopy Sciences) overnight for infiltration, and then polymerized in a 60°C oven overnight. The sections were cut on a Leica UCT ultra microtome at 80 nm using a Diatome diamond knife. Sections were contrasted with uranyl acetate and Sato's lead citrate (Electron Microscopy Sciences), and viewed and photographed on a JEOL 100CXII TEM at 60 KV (J.E.O.L. Ltd., Tokyo, Japan).

GROWTH OF HUMAN PANCREATIC CANCER CELL XENOGRAFTS IN ATHYMIC NUDE MICE

All procedures involving the use of live animals as described in this study were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center, and the NIH guidelines for humane treatment of animals were followed. Female athymic nu/nu mice, 4–5 weeks of age, were obtained from Harlan Laboratories (Indianapolis, IN). They were exposed to a 12-h light/12-h dark cycle, and had free access to a Harlan Teklad Rodent Diet 8604 (Harlan Teklad, Madison, WI) and water. Mice were housed under aseptic conditions (positive air pressure in a designated mouse room, with microisolator tops) and all mouse handling procedures were carried out under a laminar flow hood. MIA-PaCa-2 cells (5×10^6 cells/100 μl PBS) were injected s.c. into the right and left flanks of each mouse. Two weeks after inoculation, mice were randomly grouped according to body weight and tumor size and started to receive the experimental diets: Group 1 (control diet, n = 6) or Group 2 (fish oil diet, n = 6). These diets contained similar quantities of carbohydrates, protein, lipids, vitamins, and minerals, and the only difference was the type of lipids (i.e., corn oil vs. fish oil). Composition of the experimental diets used in this study is shown in Table I. Both diets were stored in sealed containers at −20°C to reduce spontaneous lipid peroxidation.

The maximum and minimum diameters of the tumors were measured once a week using a slide caliper. Tumor volume was calculated using the formula $[\pi/6 \times d^3]$, where d is the mean diameter. Body weight of each mouse was measured twice a week.

TABLE I. Composition of the Experimental Diets Used in This Study

	Control diet	Fish oil diet
Casein (g/kg)	200.0	200.0
DL-methionine (g/kg)	3.0	3.0
Sucrose (g/kg)	500.0	500.0
Corn starch (g/kg)	150.0	150.0
Corn oil (g/kg)*	50.0	–
Fish oil (g/kg)*	–	50.0
Cellulose (g/kg)	50.0	50.0
Mineral mix, AIN-76 (170915; g/kg)	35.0	35.0
Vitamin mix, AIN-76A (40077; g/kg)	10.0	10.0
Choline bitartrate (g/kg)	2.0	2.0
Ethyoxyquin, antioxidant (g/kg)	0.01	0.01

The control diet mainly consisted of linoleic acid (at 3.7 ± 0.4 mg/100 mg diet), with negligible amount of arachidonic acid. In comparison, the fish oil-supplemented diet contained EPA and DHA at 0.7 ± 0.1 and 0.5 ± 0.0 mg/100 mg diet, respectively, whereas the levels of linoleic acid and arachidonic acid were below the detection limit (Kang et al., 2010).
*Their content differs in these two diets.

At the end of the experiment, the mice were killed with CO_2 overdose followed by cervical dislocation. Tumor tissues from each mouse were removed, trimmed of the surrounding connective tissue, and weighed. The tumor tissue samples were used for various morphological and histopathological analyses as described below.

MORPHOLOGICAL AND HISTOPATHOLOGICAL ANALYSES

Tumor samples were fixed in 10% buffered formalin phosphate (Fisher Scientific, Pittsburgh, PA), dehydrated, embedded in paraffin, sectioned in 5-μm thickness, and stained with hematoxylin and eosin (H/E).

For immunohistochemical staining of the proliferating cell nuclear antigen (PCNA), 3-nitroso-cysteine, and LC3B, the antigen retrieval procedures were performed by placing the slides in 10 mM citrate buffer (pH 3.0) and heating them in a microwave oven for 20 min. After the slides were slowly cooled to room temperature, they were rinsed once with PBS, and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in PBS for 30 min and followed by rinse with PBS three times. Nonspecific binding was blocked by incubating the slides for 30 min in 2% normal goat serum (Vector Laboratories) in 1% Triton X-100 containing PBS, followed by incubation with specific antibodies against PCNA (1:500 dilution, Abcam, Cambridge, MA), 3-nitroso-cysteine (1:200 dilution, Sigma), or LC3B (1:400 dilution, Cell Signaling) in the blocking solution as described above. Then, slides were incubated with biotinylated goat anti-rabbit IgG (dilution, 1:500, Vector Laboratories) in the blocking solution. Next, the slides were incubated for 2 h with the avidin–biotin peroxidase complex (Vector Laboratories) according to the manufacturer's instructions, followed by 5 min incubation with a DAB substrate kit (Vector Laboratories). Counterstaining was performed using Mayer's hematoxylin. Negative controls lacking the primary antibody were also performed for each staining for comparison. To perform quantitative analysis of the immunostains, three to four sections per tumor tissue were selected and images were captured and analyzed using the Axiovision imaging analysis software. One field (100 μm × 100 μm) mm) in each slide was selected for quantification, and the intensity of immunoreactivity was evaluated according to the relative optical density value.

Similarly, to detect the DNA degradation in tumor tissue slides, we used the terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end labeling (TUNEL) method, that is, the ApopTag® Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA).

ANALYSIS OF PLASMA AND TISSUE LEVELS OF FATTY ACIDS BY GC–MS

Plasma and tissues (spleen, lung, intestine, kidney, pancreas, liver, heart, and brain) were obtained from each nude mouse, snap-frozen in liquid nitrogen, and stored in a −80°C freezer until analysis. The extraction and measurement of EPA and DHA in plasma and tissues were carried out according to methods described earlier [Folch et al., 1957; Morrison and Smith, 1964; Lin and Salem, 2007]. Briefly, total plasma lipids were extracted using the chloroform: methanol mixture (2:1, v/v), dried under a stream of nitrogen, and then transmethylated using boron trifluoride in methanol (14 g/L). Fatty acid methyl esters were extracted from the mixture with pentane containing 0.05% butylated hydroxytoluene. One microliter of transmethylated sample was injected into the Agilent gas chromatography 6890N linked with the 5975B mass spectrometer. Capillary column HP5-MS (30 m × 0.25 mm, 0.25 μm film thickness) was used for separation of fatty acids and helium as the carrier gas. The column oven temperature was set at 120°C, ramped to 250°C at 3°C/min, then further ramped to 300°C at 10°C/min, and held at 300°C for 5 min.

DATA ANALYSIS

The quantitative data were expressed as means ± SD. Statistical significance was determined using the analysis of variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment. P values of <0.05 were considered statistically significant.

RESULTS

MECHANISM BY WHICH EPA INDUCES THE DEATH OF CULTURED HUMAN PANCREATIC CANCER CELLS

To determine the anticancer actions of EPA and its analog DHA in human pancreatic cancer cells, two commonly used human pancreatic cancer cell lines, namely, MIA-PaCa-2 and Capan-2 cells, were used as the in vitro models. Both EPA and DHA were found to suppress the viability (based on MTT assay) of these two pancreatic cancer cell lines in vitro (Fig. 1A). The loss of cell viability following treatment with EPA or DHA was also confirmed with morphological analysis of the treated cells (data not shown). Notably, the anticancer effect of EPA in MIA-PaCa-2 cells was not affected by the co-presence of 5–20 μM of ketoprofen (a selective cyclooxygenase II inhibitor) or of aspirin (a non-selective cyclooxygenase inhibitor; data not shown), suggesting that its anticancer effect is not due to alterations in the formation of cyclooxygenase-mediated products.

To investigate the cellular mechanism by which EPA induces pancreatic cancer cell death, we focused on examining intracellular ROS accumulation and caspase activation. Recently, we showed that EPA and DHA can induce apoptosis in MCF-7 human breast cancer cells through ROS accumulation and caspase-8 activation [Kang et al., 2010]. In this study, we showed that treatment with EPA increased intracellular ROS accumulation in MIA-PaCa-2 cells in a time-dependent manner (Fig. 1B). To provide further confirmation of role of ROS accumulation in mediating pancreatic cancer cell death, we examined the effect of α-tocopherol (vitamin E), a well-known antioxidant. Co-treatment of cells with α-tocopherol and EPA almost completely suppressed intracellular ROS accumulation and cell death (Fig. 1B,C). These data indicate that accumulation of intracellular ROS following exposure to EPA contributes critically to the suppression of pancreatic cancer cell growth.

In addition, we found that the active forms (cleaved forms) of caspase-8, caspase-9, caspase-3, and poly(ADP-ribose)polymerase (PARP) were increased in EPA-treated pancreatic cancer cells (Fig. 2A). Co-treatment of these cells with z-IETD-fmk (a caspase-8 inhibitor), z-LEHD-fmk (a caspase-9 inhibitor), or z-DEVD-fmk (a caspase-3 inhibitor) each prevented them from undergoing EPA-induced cell death (Fig. 2B).

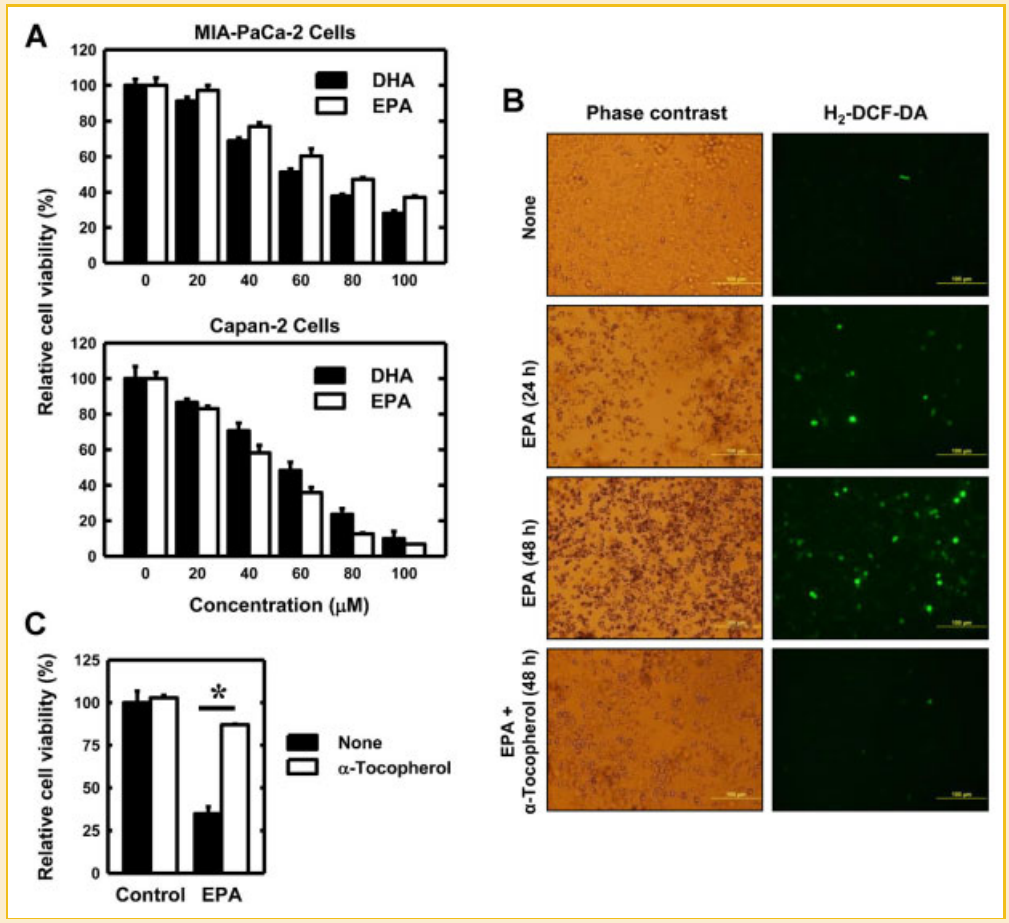

Fig. 1. Effect of EPA and DHA on cell viability and ROS accumulation in cultured human pancreatic cancer cells. A: MIA-PaCa-2 and Capan-2 cells were treated with DHA or EPA at indicated concentrations for 3 days. Gross cell viability was estimated using the MTT assay. Each value is the mean ± SD ($n = 6$). B: MIA-PaCa-2 cells were treated with 100 μM EPA alone or in the co-presence of 5 μM α-tocopherol for indicated lengths of time. Cells were then stained with 10 μM H_2-DCF-DA and analyzed for intracellular ROS accumulation using fluorescence microscopy. C: MIA-PaCa-2 cells were pre-treated with 5 μM α-tocopherol for 2 h and then 100 μM EPA was added. After 3 days of culture, cell viability was determined using the MTT assay. $^*P < 0.05$ compared to the corresponding control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

ROLE OF AUTOPHAGY INDUCTION BY EPA IN MODULATING THE DEATH OF CULTURED HUMAN PANCREATIC CANCER CELLS

While ROS accumulation in cancer cells often induces apoptosis, recent studies have shown that ROS can also induce autophagy under certain conditions [Yu et al., 2006; Scherz-Shouval et al., 2007]. Autophagy is orchestrated by a number of highly conserved autophagy-related genes (ATGs) [Reggiori and Klionsky, 2005; Rosenfeldt and Ryan, 2009]. It is known that three human LC3 isoforms (LC3A, LC3B, and LC3C) undergo post-translational modifications during autophagy. We found that the anti-LC3B antibody can strongly react with LC3B-II (i.e., the Type-II form of LC3B protein). Since conversion of LC3B-I to LC3B-II is commonly used as an indicator for autophagy formation, next we sought to determine whether EPA treatment induces autophagy by monitoring cells with LC3B-positive staining. While no LC3-positive cells were detected in vehicle-treated cells, treatment with EPA for 24-h increased LC3B-possitive cells (Fig. 3A). The LC3B-positive cells showed punctured staining in their cytosolic compartment, which is a characteristic staining pattern indicating autophagosome formation (Fig. 3B,C). The induction of autophagy by EPA was confirmed by electron microscopy analysis. Autophagosome-like structures could be readily detected in EPA-treated cells (Fig. 3D).

Beclin 1 is an important autophagy-regulated gene and required for the initiation of autophagosome formation [Sinha and Levine, 2009]. To investigate whether the induction of autophagy contributes to the induction of pancreatic cancer cell death, we selectively knocked down Becline 1 expression using Beclin 1-specific siRNAs. Beclin 1 protein expression was suppressed following transfection with the Beclin 1 siRNAs, but not with the control siRNAs (Fig. 3E). Under these conditions, EPA-induced cell death was significantly enhanced in cells transfected with Beclin 1 siRNAs (Fig. 3F). In addition, we also studied the effect of autophagy formation on EPA-induced cell death using some of the well-known autophagy inhibitors (e.g., chloroquin or bafilomycin A). We found that pharmacologic inhibition of autophagosome formation by these inhibitors enhanced EPA-induced cell death (Fig. 3G). These data indicate that the concomitant induction of autophagy by EPA

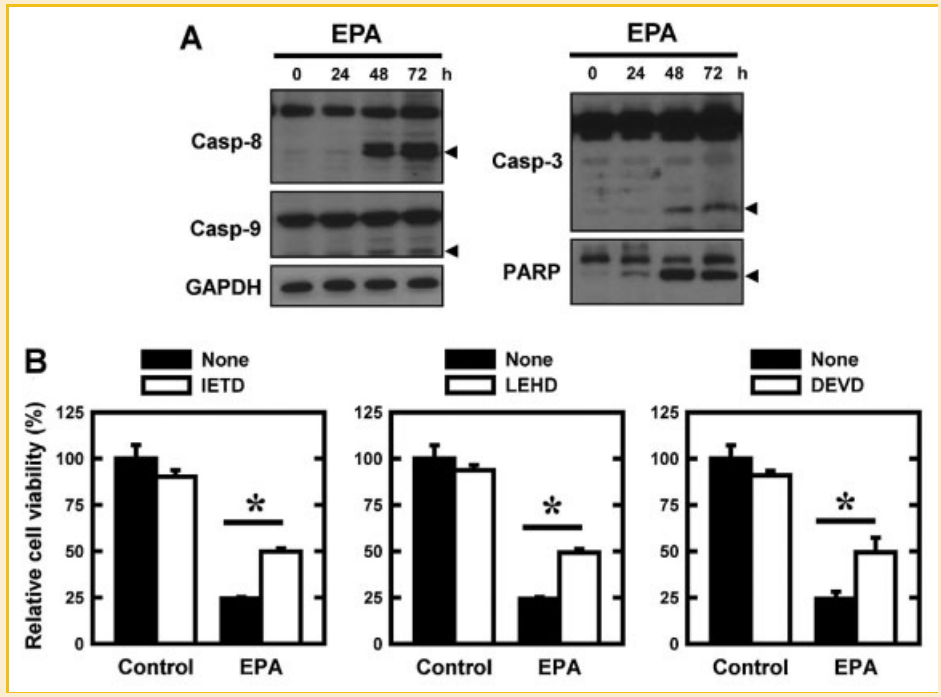

Fig. 2. EPA induces activation of caspase-8, -9, -3, and PARP in cultured human pancreatic cancer cells. A: MIA-PaCa-2 cells were treated with 100 μM EPA for indicated lengths of time, and then cell extracts were prepared and subjected to Western blotting analysis. Arrows point to the cleaved forms of caspase-8, -9, -3, and PARP. B: MIA-PaCa-2 cells were pre-treated with 20 μM of each caspase inhibitor for 2 h and then 100 μM EPA was added. Here IETD (z-IETD-fmk) was used as a caspase-8 inhibitor, LEHD (or z-LEHD-fmk) as a caspase-9 inhibitor, and DEVD (z-DEVD-fmk) as a caspase-3 inhibitor. After 3 days of culture, cell viability was analyzed using the MTT assay. $^{*}P < 0.05$ compared to the corresponding control.

in MIA-PaCa-2 cells partially suppresses its ability to induce apoptotic cell death.

SUPPORT FOR THE IN VITRO MECHANISTIC FINDINGS IN THE IN VIVO TUMOR XENOGRAFT MODEL

First, to evaluate the in vivo anticancer efficacy of omega-3 FAs on human pancreatic cancer, we used the growth of MIA-PaCa-2 cancer cell xenografts in athymic nu/nu mice as a representative in vivo model. Each mouse was injected s.c. with MIA-PaCa-2 cells (5×10^6 cells in 100 μL PBS), one in the left flank and one in the right flank. Two weeks later, the mice were randomly grouped, and started to receive the experimental diets. The animals in Group 1 were fed the control diet, and animals in Group 2 were fed the 5% fish oil-supplemented diet. These two diets contained the same quantities of carbohydrates, protein, lipids, vitamins, and minerals (summarized in Table I), and the only difference is the type of lipids (i.e., corn oil vs. fish oil). No significant difference was observed in animal body weight change between animals in these two groups (Fig. 4A). Also, the amount of food intake between these two groups was not significantly different throughout the experiment (Fig. 4B). However, treatment with 5% fish oil-supplemented diet significantly suppressed tumor growth and tumor weight gain (Fig. 4C,D).

To determine whether the mechanism of omega-3 FA-induced cell death observed in vitro also can be seen in vivo, we analyzed the dissected tumor tissues using immunohistochemical staining. While the morphology and density of tumor cells were not significantly different (Fig. 5; H/E staining), the number of PCNA-positive cells was decreased in the fish oil diet group, suggesting that there was a decrease in cancer cell proliferation (Fig. 5; PCNA staining). In addition, the number of TUNEL-positive cells was significantly increased in the fish oil diet group, suggesting that there was an increase in cancer cell death (Fig. 5; TUNEL staining). The levels of 3-nitroso-cysteine residues in cellular proteins, a marker for protein nitration and cellular oxidative stress, were also determined in tumor cell xenografts. We found that the 3-nitroso-cysteine level was significantly elevated in tumor tissues in the fish oil diet group (Fig. 5; 3-nitroso-cysteine staining). Similar changes in the 3-nitrotyrosine immunostaining (another marker of cellular oxidative stress based on detection of protein nitrosylation) were also observed (data not shown). Furthermore, the number of LC3B-positive cells was increased in the fish oil diet group (Fig. 5; LC3B staining), suggesting an increase in autophagosome formation in tumor cells from fish oil-treated animals. While more studies are needed, these data show that the proposed mechanism for the anticancer actions of EPA based on in vitro studies are in agreement with the observations made with the in vivo tumor samples.

PLASMA AND TISSUE LEVELS OF EPA AND DHA AFTER ORAL FEEDING OF THE FISH OIL DIET

The plasma and tissue levels of EPA and DHA were determined in mice orally fed the 5% fish oil-supplemented diet. Both DHA and EPA concentrations in plasma were increased in fish oil diet group (Fig. 6A). EPA tissue level was significantly elevated in most tissues, and the increase of EPA level in the pancreas was most pronounced

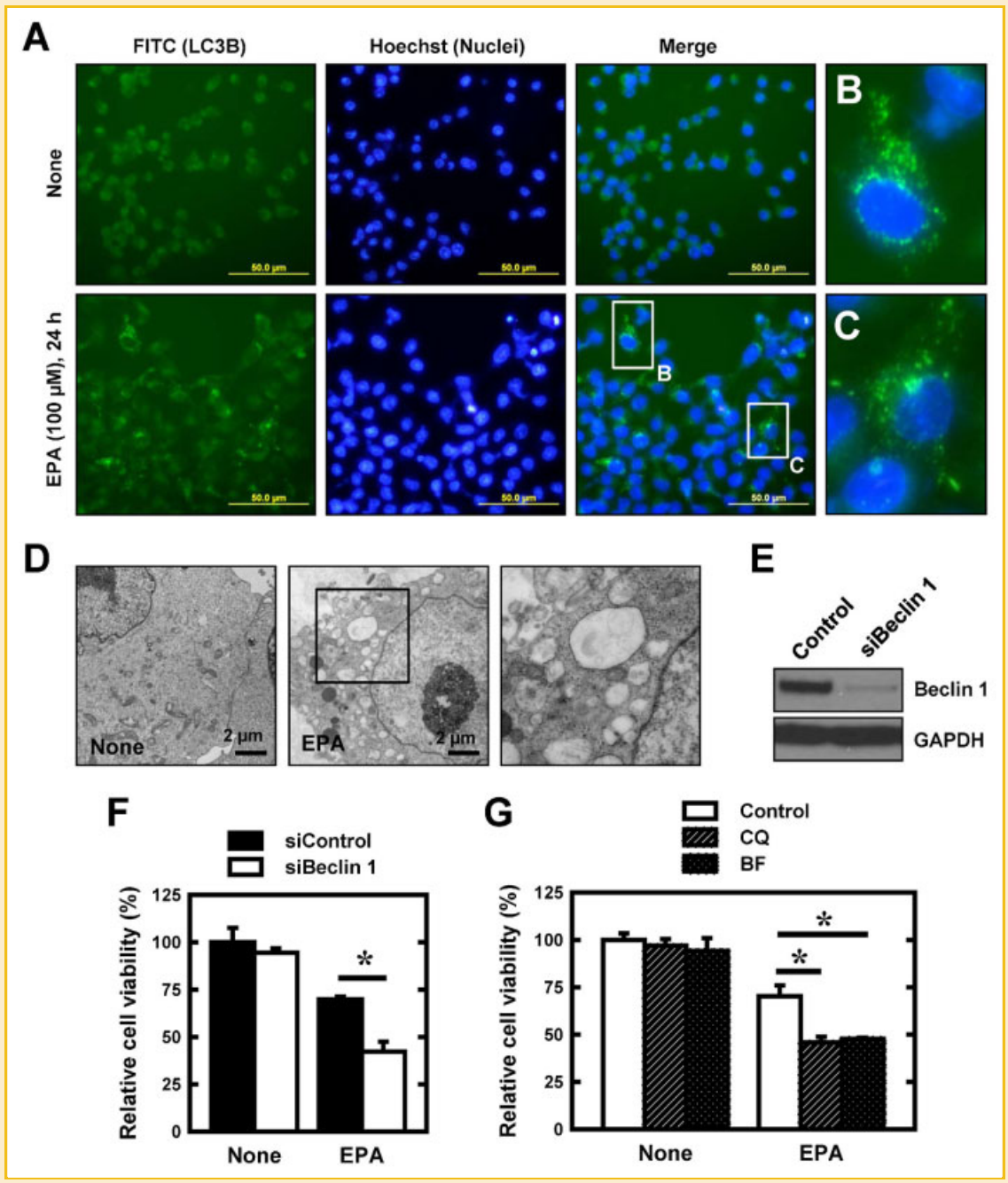

Fig. 3. EPA induces autophagy in cultured human pancreatic cancer cells. A: MIA-PaCa-2 cells were treated with 100 μM EPA for 24 h. Cells were then fixed and analyzed for the immunefluorescence staining of LC3B. Nuclei were stained with Hoechst-33342. B,C: Enlarged images from EPA-treated cells. D: MIA-PaCa-2 cells were treated with 100 μM EPA for 24 h, and then the cells were fixed and analyzed for autophagosome formation using the transmission electron microscopy (TEM). E: MIA-PaCa-2 cells were transfected with scrambled siRNAs or specific Beclin-1 siRNAs. After 24 h of incubation, cell extracts were prepared and subjected to Wstern blotting analysis using the Beclin-1-specific antibody. The membrane was stripped and re-probed for GAPDH as a loading control. F: MIA-PaCa-2 cells were transfected with scrambled or specific Beclin-1 siRNAs. Twenty-four h later, the cells were treated with 100 μM EPA for additional 48 h. Cell viability was determined using the MTT assay. G: MIA-PaCa-2 cells were first treated with 5 μM chloroquine (CQ) or 5 nM bafilomycin A (BF) for 2 h and then 100 μM EPA was added. After 2 days of culture, cell viability was analyzed using the MTT assay. $^{*}P < 0.05$ compared to the corresponding control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

(Fig. 6B). By contrast, the differences in DHA tissue levels between the control and fish oil diet groups were rather modest. In addition, it was observed that the EPA and DHA levels in cancer xenografts were increased by fivefold and twofold, respectively, in fish oil diet-fed animals (Fig. 6C).

DISCUSSION

The concept of using natural food chemicals such as EPA and DHA as cancer chemopreventive and therapeutic agents is attractive because these chemicals are usually available at a low cost, can be

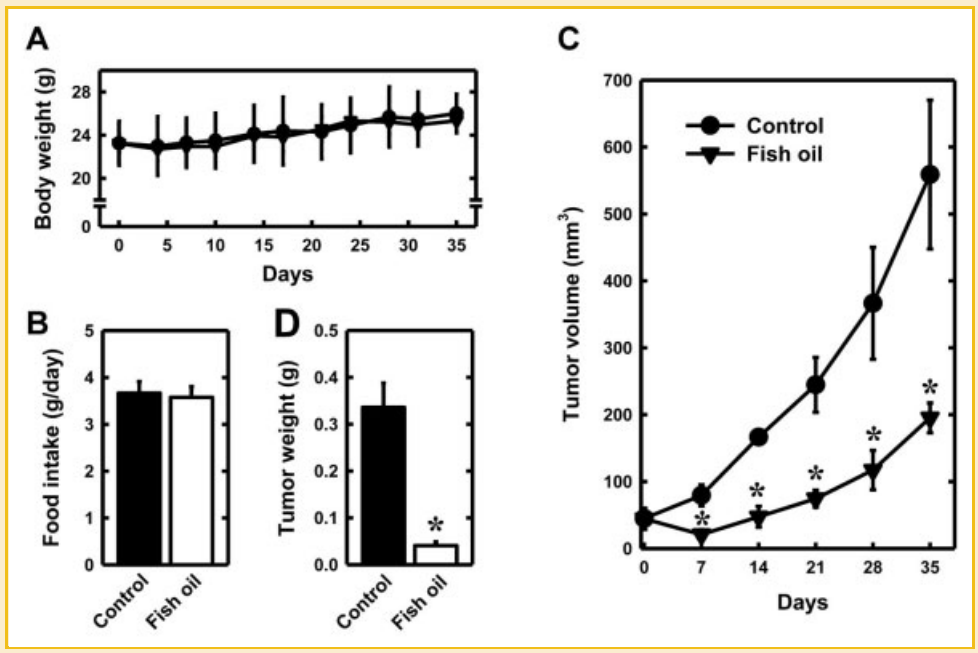

Fig. 4. Effect of 5% fish oil-supplemented diet on the growth of MIA-PaCa-2 human pancreatic cancer cell xenografts in athymic nude mice. A: MIA-PaCa-2 cells (5×10^6 cells/100 μL PBS) were s.c. injected into athymic nude mice as described in the Materials and Methods Section. After injection of the cancer cells, the animals were first maintained on a control diet for two weeks, and then they were started to be fed a control diet or a fish oil diet for additional five weeks. The changes in body weight (A) and amount of food intake (B) was measured once a week (C) were measured twice a week, and the change in tumor growth. D: At the end of the experiment, each tumor was removed, trimmed, and weighed. $^*P < 0.05$ compared to the control diet group.

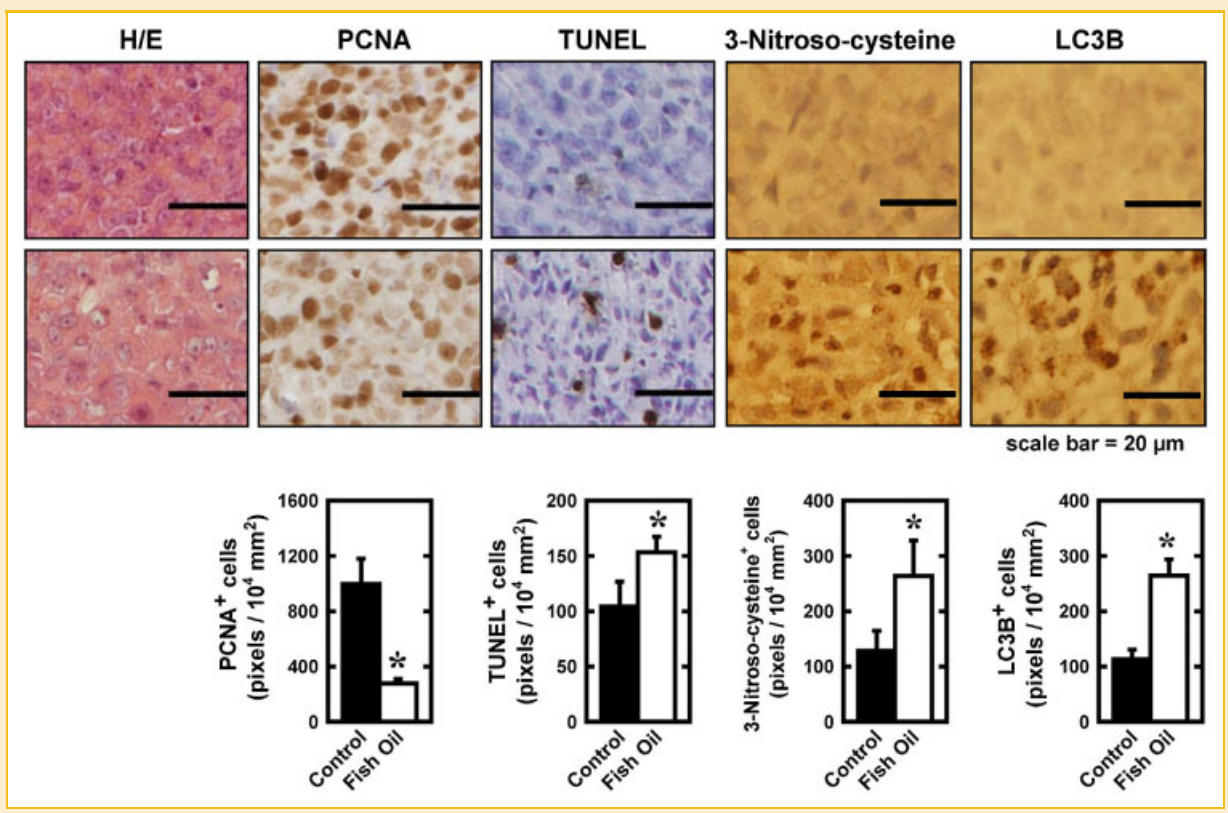

Fig. 5. Histopathological analysis of human pancreatic cancer cell xenografts in athymic nude mice. The experimental details are the same as described in the legend to Figure 4. Tumor samples that were removed from each mouse at the end of the experiment were processed for regular H/E staining as well as for analysis of cells that are immunohistochemically stained positive for PCNA, TUNEL, 3-nitroso-cysteine, and LC3B. The quantitative data are shown in the lower panels. $^*P < 0.05$ compared to the control diet group. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

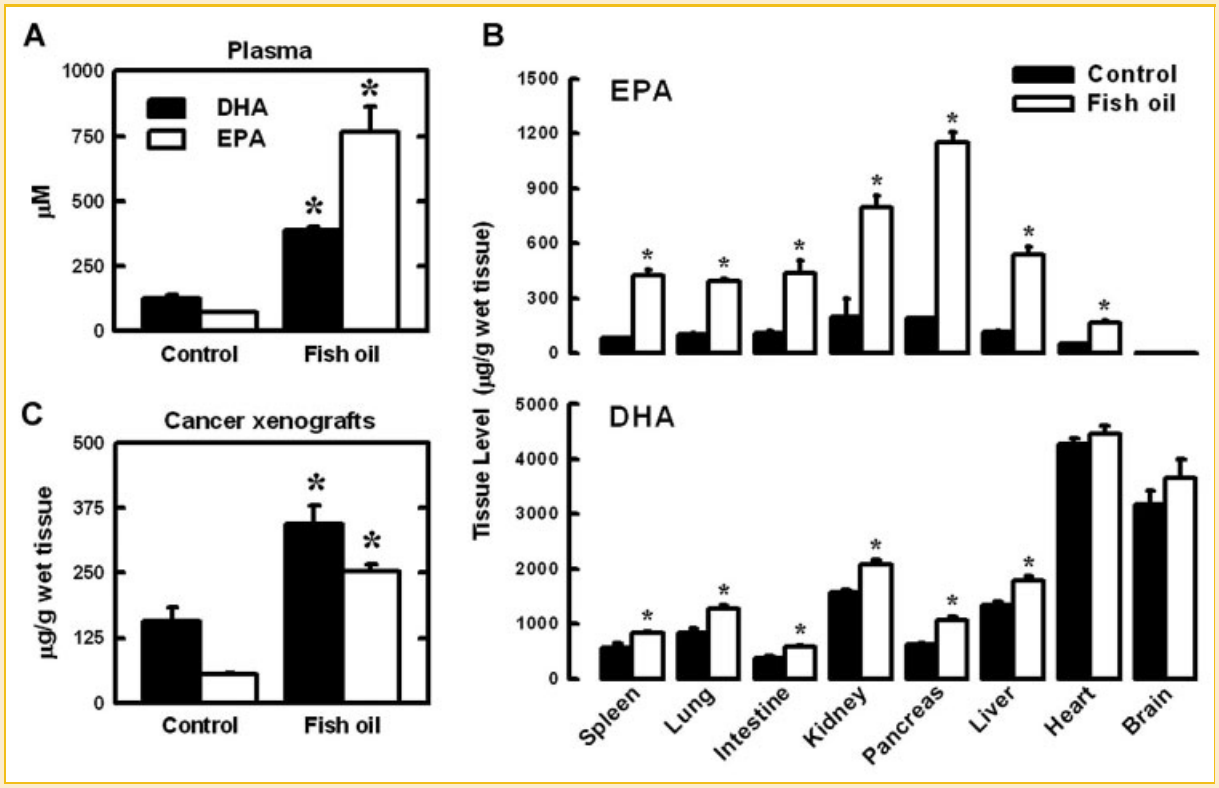

Fig. 6. EPA and DHA levels in plasma, tissues, and pancreatic tumor xenografts after feeding mice the 5% fish oil–supplemented diet. A,B. Comparison of the concentrations of EPA and DHA in plasma (A) and various tissues (B). Comparison of the concentrations of EPA and DHA in cancer xenografts (C). The data in panels A and B were obtained using control (tumor-free) athymic nude mice that were fed either the control diet or the 5% fish oil–supplemented diet for 2 weeks, whereas the data shown in panel C were obtained using the tumor xenografts collected at the end of the anticancer experiment as shown in Figure 4. Analysis of the fatty acid levels was carried out using GC-MS as described in the Materials and Methods Section. $^{*}P < 0.05$ compared to the control diet group.

readily metabolized in human body, and usually do not produce significant harms to normal cells [Arai, 1996; Murakami et al., 1996]. Earlier studies reported that PUFAs have little or no toxicity in normal cells [Anel et al., 1992; Bégin et al., 1985]. In a recent study, we reported that EPA and DHA, two common omega-3 FAs, can cause ROS accumulation and subsequently induce caspase-8-dependent apoptosis in human breast cancer cells both in vitro and in vivo (as cancer xenografts in athymic nude mice) [Kang et al., 2010]. Based on the in vivo pharmacokinetic results obtained in this study showing that the pancreas has a unique ability to accumulate EPA at a level markedly higher than several other tissues, we sought to further investigate the anticancer actions of EPA and its analog DHA in human pancreatic cancer cells using both in vitro and in vivo models. We found that EPA and DHA can strongly induce cell death in cultured human pancreatic cancer cells (MIA-PaCa-2 and Capan-2). In addition, feeding animals a diet supplemented with 5% fish oil, which contains high levels of EPA and DHA, strongly suppresses the growth of MIA-PaCa-2 human pancreatic cancer xenografts in athymic nude mice, by inducing apoptotic cell death. Notably, chronic feeding of animals with 5% fish oil diet did not alter the animal body weight compared to control diet-fed animals, suggesting that the fish oil diet has little or no gross toxicity in these animals.

Mechanistically, the cell death induced by DHA is largely mediated by increased production of ROS in cancer cells. The crucial role of ROS in DHA-induced death of human pancreatic cancer cells is supported by the following observations made in the present study: First, there was an increase in the intracellular ROS accumulation in cultured MIA-PaCa-2 cells following treatment with EPA. Second, the cytotoxicity induced by EPA in these cells was effectively protected by the co-presence of an antioxidant. Third, using the cellular levels of 3-nitroso-cysteine as a marker for protein nitration and cellular oxidative stress in vivo, we showed that there was a marked increase in ROS level in tumor tissues obtained from fish oil diet-fed animals compared to control diet-fed animals. Together, these data support the suggestion that EPA-induced cell death is largely mediated through the formation of intracellular ROS.

By using the annexin-V/PI double staining and TUNEL assay, we confirmed that EPA can induce apoptosis both in vitro and in vivo. In addition, we found that caspase-8, an effector caspase, is activated in EPA-treated cells, and the use of a pharmacological inhibitor of caspase-8 protects the cells from EPA-induced cell death. Pharmacological inhibition of caspase-9 or caspase-3 also exerts a protective effect. Because caspase-9 and -3 are known to be activated following caspase-8 activation in MIA-PaCa-2 cells, which are Type II cells, it is thus postulated that caspase-8 activation is an initiating event in DHA-induced apoptotic cell death.

At present, the precise mechanism by which EPA selectively activates caspase-8 is not understood. It is speculated that EPA may

preferentially increase ROS accumulation in or near the plasma membrane lipid rafts where the assembly of the death-inducing signaling complex (DISC) and the subsequent activation of caspase-8 takes place [Gajate et al., 2009].

In this study, we found that EPA not only induces the caspase-8-dependent apoptosis in human pancreatic cancer cells, but it also induces autophagy. There is still considerable controversy regarding the precise role of autophagy in cell death [Kroemer and Levine, 2008]. Autophagy is known to enhance cell survival under certain conditions, such as in response to nutrient deprivation, organelle damage, or other stresses. However, excessive or prolonged autophagy may also induce cell death. For example, studies using embryonic fibloblasts from Bax/Bak double knockout mice showed that while these cells are resistant to apoptosis, they can still undergo a non-apoptotic cell death after death stimulation [Shimizu et al., 2004]. This form of cell death has been suggested to be autophagic cell death [Shimizu et al., 2004]. In this study, we detected autophagosome formation at 24 h after EPA treatment, while caspase activation was not observed at that time point. These observations suggest that the EPA-induced autophagy in pancreatic cancer cells occurs before caspase activation and apoptosis induction in pancreatic cancer cells. To probe the role of autophagy in EPA-induced cell death, we inhibited the autophagy signaling pathway using two different methods, that is, transfection with the Beclin 1 siRNAs and the use of pharmacological inhibitors against autophagosome formation. In both cases, EPA-induced cell death was significantly enhanced. Therefore, it is concluded that autophagy induction by EPA partially inhibits or delays the induction of apoptosis in human pancreatic cancer cells. Notably, the concomitant induction of apoptosis and autophagy was also detected in human pancreatic cancer xenografts in athymic nude mice treated with a diet supplemented with fish oil. We could readily detect increases in both TUNEL-positive cells (for dying cells) and LC3B-positive cells (for autophagic cells) in cancer xenografts in fish oil diet-fed animals.

It is estimated that while an average American typically consumes 0.7–1.6 g of n − 3 PUFAs each day [Conquer and Holub, 1998; Kris-Etherton et al., 2000], the Greenland Inuit may consume 6–14 g per day, which corresponds to 2.7–6.3% of daily energy [Feskens and Kromhout, 1993; Damsgaard et al., 2008]. Traditional Japanese diets contain 1–2% of daily energy as long chain n − 3 PUFAs [Okuyama et al., 1996; Nagata et al., 2002]. In various human clinical trials, administration of 1–9 g of n − 3 PUFAs per day (0.45–4% of total calories), mainly in the form of EPA and DHA, have been widely used [Kelley et al., 1998; Kelley et al., 1999; Thies et al., 2001; Rees et al., 2006]. The amount of 5% (w/w) fish oil diet, which contains approximately 1.2% purified omega-3 FAs in the diet, is within the acceptable range consumed by humans and used in human clinical trials. When the animals were chronically fed a diet supplemented with this level of fish oil, little or no gross toxicity was observed.

It is of interest to note that when the mice were fed the 5% fish oil-supplemented diet, both EPA and DHA concentrations in their plasma are markedly increased. However, only the levels of EPA, but not DHA, were markedly increased in most tissues, and the increase of EPA in the pancreas was most pronounced among the tissues tested. Similarly, in pancreatic cancer xenografts, EPA level was increased approximately fivefold, whereas DHA level was only approximately doubled.

Although it is well known that different tissues have a very different ability to accumulate certain fatty acids, the exact mechanism for their differential distribution in different tissues/cells is not clearly understood at present. Since multiple transporter proteins are involved in the cross-membrane transport of various fatty acids [Dutta-Roy, 2000a,b; Gil-Sánchez et al., 2012], it possible that the differential distribution of these transporter proteins may be partly responsible for the differential tissue distribution of certain fatty acids.

In summary, EPA can induce apoptotic cell death in human pancreatic cancer cells in vitro and in vivo, and this is the main mechanism of their anticancer actions. In addition, EPA can also induce autophagy in these cancer cells. It is suggested that induction of autophagy by EPA would inhibit its induction of apoptotic cell death. More studies are needed to further elucidate the precise role of autophagy induction in EPA-induced apoptotic cancer cell death in vivo, which will aid in the design of more effective strategies for using EPA in the treatment of human pancreatic cancer.

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