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Increased lipid metabolism and cell turnover of MiaPaCa2 cells induced by high-fat diet in an orthotopic system

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Abstract

In this study, we investigated whether increased dietary fat influences established pancreatic cancer cells. MiaPaCa2 human pancreatic cancer cells were grown orthotopically in athymic mice fed normal diet (ND) or high-fat diet (HF). In the resulting tumors, medium-chain acyl–coenzyme A dehydrogenase (MCAD, a regulator of fatty acid β-oxidation) and Cu/Zn–superoxide dismutase (an antioxidant enzyme) were determined using Western blotting. The MCAD messenger RNA (mRNA) was determined by real-time polymerase chain reaction. Intracellular lipid droplets, proliferating cells (Ki67 positive), and apoptotic cells were stained in tumor sections. The HF tumors were heavier than the ND tumors (1.60 ± 0.08 vs 1.13 ± 0.10 g, $P < .01$, 6 tumors per group). The MCAD and Cu/Zn–superoxide dismutase proteins and the MCAD mRNA were increased in HF tumors compared with those seen in ND tumors. The HF tumors contained extensive central necrosis, which was surrounded with apoptotic and proliferating cells. The HF tumors also showed numerous lipid droplets. In the ND tumors, necrosis was uncommon, apoptotic cells were sporadic, and lipid droplets were few. In follow-up experiments, MiaPaCa2 cells were incubated in vitro in the presence or absence of fatty acids (oleic and linoleic acids). The fatty acid exposure increased lipid droplets, cell proliferation, and MCAD mRNA expression in MiaPaCa2 cells. In conclusion, increased dietary fat stimulates lipid metabolism and cell turnover in MiaPaCa2 human pancreatic cancer cells.

1. Introduction

High-fat diets increase the expression of enzymes such as acyl–coenzyme A (CoA) dehydrogenases that are involved in β-oxidation of fatty acids [1]. As a result, more fatty acids are metabolized as energy substrates. In addition, high-fat diets may increase reactive oxygen species (ROS) and alter the expression of antioxidant enzymes such as superoxide dismutases [2,3]. High-fat diets may also increase intracellular lipid droplets [4] and plasma free fatty acids [1].

Epidemiologic studies have shown that high-fat diets are a risk factor for pancreatic cancer in humans [5]. High-fat diet increased the growth of SW-1990 human pancreatic cancer cells as subcutaneous tumors in athymic mice [6]. In a hamster model of pancreatic cancer induced by the carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP), high-fat diets stimulated the process of carcinogenesis [7,8]. In rats, a high-fat/high-protein diet increased pancreatic carcinogenesis induced by another chemical carcinogen [9]. In these rodent models of carcinogen-induced pancreatic cancer, it is difficult to tell whether increased dietary fat exerts its effects when the first cancer cells are forming or in later stages after the first cancer cells have already formed. For the investigators whose interest is to see whether dietary fat affects established pancreatic cancer cells, transplantation of human pancreatic cancer cells into the pancreas of athymic mice is a good model. To our knowledge, this model has not been used previously in any high-fat–diet studies.

We undertook this study to investigate whether increased dietary fat influences established cancer cells. We implanted MiaPaCa2 human pancreatic cancer cells orthotopically in

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athymic mice that were fed high-fat diet (HF) or normal diet (ND). In the resulting tumors, we examined Cu/Zn-superoxide dismutase (SOD1), medium-chain acyl-CoA dehydrogenase (MCAD), cell proliferation, apoptosis, and intracellular lipid droplets. In follow-up experiments, lipid metabolism and cell proliferation were also studied in MiaPaCa2 cells in vitro after the cells were incubated with fatty acids. We chose the poorly differentiated cell line MiaPaCa2 for this study because these cells are transplantable in the pancreas of athymic mice [10] and are also regulated by fatty acids in vitro [11,12].

2. Materials and methods

2.1. MiaPaCa2 cells and athymic mice

MiaPaCa2 cells were bought from the American Type Culture Collection (Rockville, MD) and were cultured in 95% air and 5% CO_2 using Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum. The medium and serum were bought from Invitrogen (Stockholm, Sweden). Male athymic mice were purchased from Taconic Europe (Ry, Denmark). The Southern Stockholm Animal Research Committee approved the study. An ND and an HF were bought from Lactamin (Kimstad, Sweden). The contents of the diets are shown in Table 1. In addition, a mix of vitamins was added to both diets. As a result, each kilogram of the diets was supplemented with vitamins A (12 000 IU), B1 (3 mg), B2 (10 mg), B3 (40 mg), B5 (10 mg), B6 (4 mg), B12 (0.02 mg), D (1500 IU), E (60 mg), and K3 (10 mg). A mix of minerals was added to both diets. As a result, each kilogram of the diets was supplemented with calcium (10 g), phosphorus (8 g), potassium (6 g), sodium (3 g), magnesium (2 g), iron (0.2 g), zinc (0.1 g), and manganese (0.1 g).

2.2. Orthotopic implantation

After arrival, athymic mice (N = 20) were kept in a room with a 12-hour light/dark cycle and had free access to food (ND) and drinking water. After the animals were acclimated, half of the mice (n = 10) were fed HF for a priming period of 6 weeks. During this period, the other mice (n = 10) were still fed ND. At the end of this priming period, the body weight of HF mice showed a trend toward an increase (HF: 27.3 ± 0.7 g, n = 10 vs ND: 24.4 ± 1.3 g, n = 10; $P = .08$). The 20 mice were divided into 4 groups: HF-fed tumor carriers (HF-T, n = 6), HF-fed controls (HF-C, n = 4), ND-fed tumor carriers (ND-T, n = 6), and ND-fed controls (ND-C, n = 4).

Table 1
Mouse diets

Contents	ND	HF
Ingredients (%)		
Carbohydrate	55.7	43.9
Protein[a]	18.5	17.2
Fat[b]	4.0	21.0
Cellulose (fiber)	3.5	3.9
Ash	6.3	4.1
Water	11.9	9.8
Energy (kJ/g)	12.6	15.6

[a] Derived from soybean and wheat.
[b] Derived from soybean oil in ND and from cocoa butter in HF.

MiaPaCa2 cells were suspended in DMEM. In each mouse, 3×10^6 MiaPaCa2 cells were injected into the pancreas [13]. After the procedure, HF and ND were given to the corresponding groups for 12 weeks. At the end of the 12-week period, all the mice were anesthetized and killed by heart puncture. Blood was withdrawn from the heart. The plasma was separated. The pancreas was examined, and any visible tumors were removed.

2.3. Exposure of MiaPaCa2 cells to fatty acids in vitro

A mixture of fatty acids was bought from Sigma (St Louis, MO). This preparation contained oleic acid (800 μg/mL) and linoleic acid (800 μg/mL) dissolved in a phosphate buffer with 10% bovine serum albumin. The preparation was added to DMEM at 2 doses, giving low– and high–fatty acid media. The final concentrations of fatty acids were 40 and 160 μg/mL in these media. Control medium had no fatty acids. In each experiment, MiaPaCa2 cells were incubated in the test media for 6 hours.

2.4. Histology

Cryosections (8-μm thick) were prepared from tumor xenografts. Two basic buffers were prepared before oil red O staining, that is, 0.5% oil red O (Sigma) in isopropyl alcohol and 1% dextrin in water. Three parts of the oil red O buffer and 2 parts of the dextrin buffer were mixed. In the resulting buffer, cryosections were incubated for 20 minutes. Proliferating cells (Ki67 positive) were determined in tumor sections fixed in 1% paraformaldehyde. The sections were incubated with an anti-Ki67 antiserum (Dako [Glostrup, Denmark] M7248, 1:100) for 30 minutes at room temperature and then processed using a Vector ABC immunocytochemical kit (Burlingame, CA). In a terminal transferase dUTP nick end labeling assay, tumor sections were fixed in 1% paraformaldehyde and processed using an ApopTag Apoptosis Detection Kit (Chemicon, Temecula, CA). All sections were counterstained with hematoxylin. Oil red O and Ki67 staining was also done in MiaPaCa2 cells after 6-hour incubation in the presence or absence of fatty acids. An author (MKB) who was blind to the treatment counted the Ki67-positive cells under a microscope.

2.5. Real-time polymerase chain reaction

Medium-chain acyl-CoA dehydrogenase messenger RNA (mRNA) was determined in a TaqMan duplex real-time polymerase chain reaction, using 18S ribosomal RNA (rRNA) as control. Total RNA was extracted from tumor xenografts and from MiaPaCa2 cells cultured in 6-well plates

Table 2
Tumor weight and plasma glucose and insulin levels

Mice	n	Diets	Tumor burden	Tumor weight (g)	Glucose (mg/dL)	Insulin (ng/mL)
ND-C	4	Normal	−		265 ± 45	0.82 ± 0.05
HF-C	4	High fat	−		347 ± 50	1.03 ± 0.15
ND-T	6	Normal	+	1.13 ± 0.10	259 ± 29	1.25 ± 0.40
HF-T	6	High fat	+	1.60 ± 0.08*	281 ± 23	1.21 ± 0.12

* *P* less than .01 compared with the values seen in the ND-T group.

[13]. Complementary DNAs were synthesized by incubating the following reagents together for 60 minutes at 42°C: 1 μg RNA, 0.5 μg oligo(dT), the primers for 18S rRNA (400 pmol/L), and a master mix containing concentrated reaction buffer, AMV reverse transcriptase (0.8 U), $MgCl_2$, and RNasin (Promega, Madison, WI). The resulting complementary DNA (50 ng) was mixed with the human MCAD TaqMan gene expression mix (assay ID: Hs00163494_m1) and the probe (500 nmol/L) and forward/reverse primers (100 nmol/L) for 18S rRNA (Applied Biosystems, Foster City, CA). The reagents went through a phase of 50°C (2 minutes) and 95°C (10 minutes) in an ABI PRISM-7000 Sequence Detection System (Applied Biosystems) and were cycled 40 times between 95°C (15 seconds) and 60°C (1 minute). The MCAD values were analyzed in relation to concurrent 18S data.

2.6. Western blotting assays

Cytosolic proteins were extracted from tumor xenografts [13] and separated on 8% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels to detect MCAD and SOD1, respectively. The proteins were transferred to polyvinylidene difluoride membranes and incubated overnight at 4°C with an anti-MCAD antiserum (Abcam [Cambridge, UK] 13677) or an anti-SOD1 antiserum (StressMarq [Victoria, BC, Canada], SPC-115C/D). The membranes were incubated for 1 hour at room temperature with a secondary antiserum (Chemicon AP106P for MCAD and Amersham [Arlington Heights, IL] NA934 for SOD1). Western blotting was visualized with electrochemiluminescence reagents and recorded on film. The staining was stripped, and the membranes were restained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using appropriate antisera (Abcam 9483 and Chemicon AP106P). All Western blotting results were scanned and then digitalized using the National Institutes of Health Image software. The MCAD and SOD1 data were related to corresponding GAPDH data to give the MCAD/GAPDH and SOD1/GAPDH ratios.

2.7. Other assays

Plasma free fatty acids and insulin were determined using a nonesterified fatty acid C kit (Wako, Richmond, VA) and a radioimmunoassay kit for rat insulin (Linco, St Charles, MO). Plasma glucose levels were measured in a biochemical analyzer (YSI, Yellow Springs, OH). Thymidine incorporation in cultured MiaPaCa2 cells was determined as previously described [13].

2.8. Statistics

Data are shown as means ± SEM. Analysis of variance followed by the Bonferroni posttest was used when 3 or more groups were compared. The Student *t* test was used when 2 groups were involved. *P* less than .05 was considered significant.

3. Results

All the mice were alive at the end of the 12-week period. Pancreatic tumors were found in all HF-T and ND-T mice and in none of the HF-C and ND-C mice. The HF tumors were significantly heavier than the ND tumors (Table 2). No significant differences among groups were seen in plasma

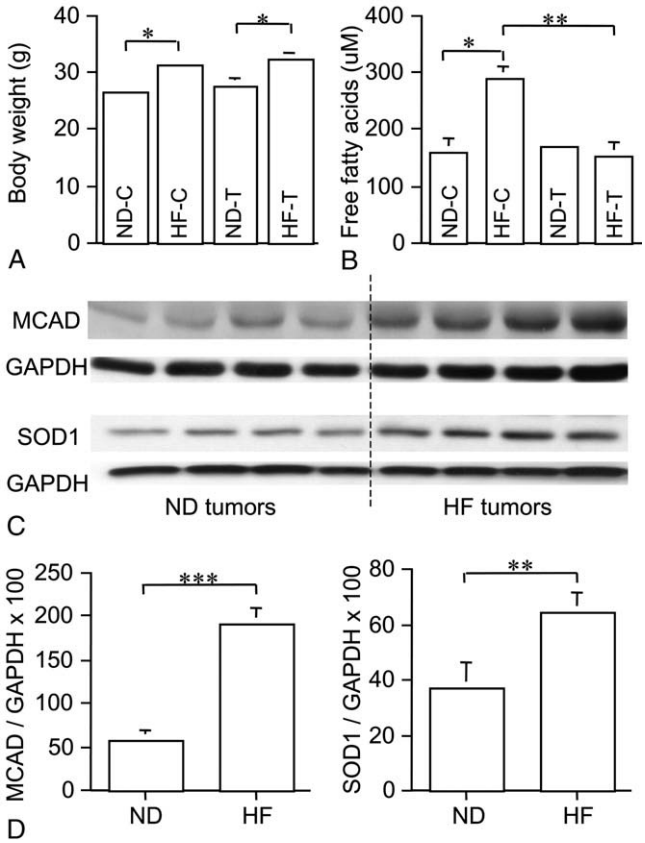

Fig. 1. MiaPaCa2 human pancreatic cancer cells were implanted orthotopically in athymic mice fed HF or ND, giving 2 groups of tumor cell carriers (HF-T, n = 6 and ND-T, n = 6). Untreated mice fed HF or ND were used as controls (HF-C, n = 4 and ND-C, n = 4). Twelve weeks later, the mice were killed. Body weight (A) and plasma free fatty acids (B) were determined. C, Medium-chain acyl-CoA dehydrogenase and SOD1 were determined in tumor grafts by Western blotting. Glyceraldehyde-3-phosphate dehydrogenase was used as the loading control. D, The results in C were digitalized and are shown as MCAD/GAPDH and SOD1/GAPDH ratios. **P* less than .05, ***P* less than .01, and ****P* less than .001.

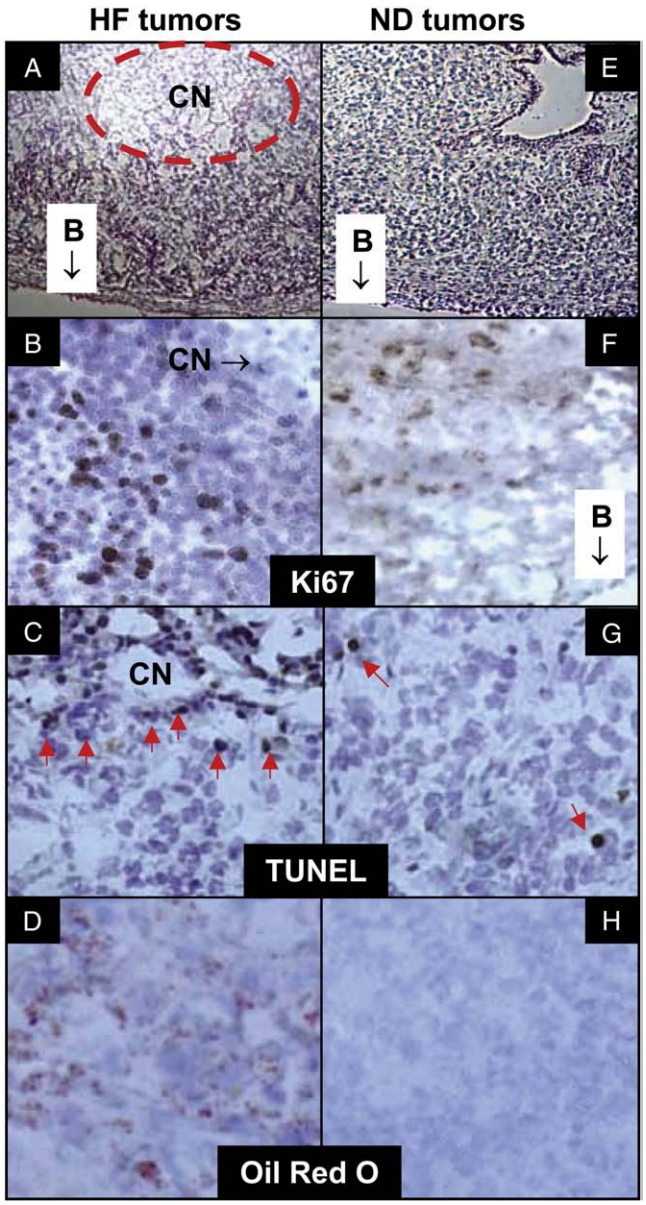

Fig. 2. Cryosections were prepared from HF (A-D) and ND tumors (E-H). A and E (100×), Sections were stained with hematoxylin. B and F (200×), The nuclei of proliferating cells (Ki67 positive) were stained in brown by immunocytochemistry. C and G (200×), Apoptotic cells were stained in brown by terminal transferase dUTP nick end labeling. Arrows indicate typical staining. The arrows in C also indicate the border of central necrosis. D and H (400×), Intracellular lipid droplets were stained as red dots by oil red O. CN indicates central necrosis; B, tumor border.

levels of glucose and insulin (Table 2). Body weights in the 2 HF groups were significantly higher than those in the respective ND groups (Fig. 1A). Plasma free fatty acid levels in HF-C mice were higher than those in ND-C and HF-T mice (Fig. 1B). However, plasma free fatty acid levels in HF-T mice were not significantly different from those seen in ND-C or ND-T mice (Fig. 1B). In the tumor analysis, HF tumors contained more MCAD and SOD1 proteins than ND tumors (Fig. 1C, D). When MCAD mRNA values were analyzed, the mean value in 6 ND tumors was used as a baseline (100%) to which the data from 6 HF tumors were related. As a result, MCAD mRNA in HF tumors was found to be increased to 133% ± 9% ($P < .05$).

Histologic features in HF tumors are shown in Fig. 2A to D. These tumors had profound central necrosis (Fig. 2A). The necrotic region was surrounded with proliferating cells (Fig. 2B). Apoptotic cells were found lined along the border of necrosis (Fig. 2C). Intracellular lipid droplets were numerous in HF tumors (Fig. 2D). In ND tumors, little or no central necrosis was seen (Fig. 2E). In these control tumors, proliferating cells were scattered in peripheral regions (Fig. 2F), as we showed previously [13]. Apoptotic cells were sporadic and scattered randomly in the ND tumors (Fig. 2G), and lipid droplets were scarce (Fig. 2H).

Fig. 3 shows Ki67 expression (A-C) and lipid droplets (D-F) in MiaPaCa2 cells that were incubated in different media. When Ki67-positive cells and lipid droplets were scored,

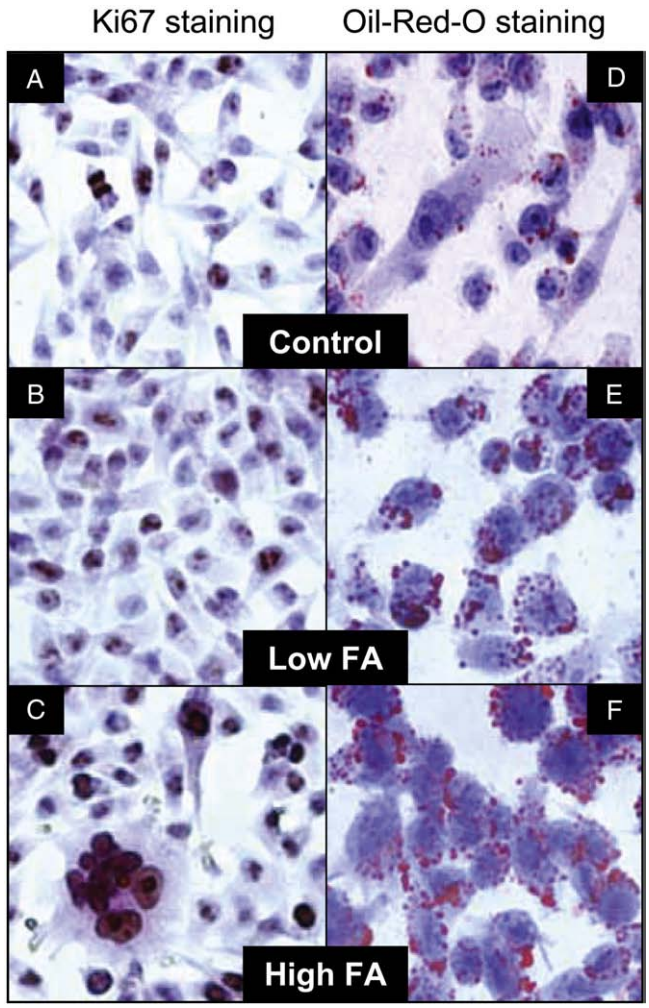

Fig. 3. A mixture of FA was added to culture medium at 2 doses, giving low-FA and high-FA media. Control medium had no FA. MiaPaCa2 cells were incubated for 6 hours in these media. Proliferating (Ki67 positive) cells were stained by immunocytochemistry (A-C, 400×). Lipid droplets were stained by oil red O (D-F, 600×).

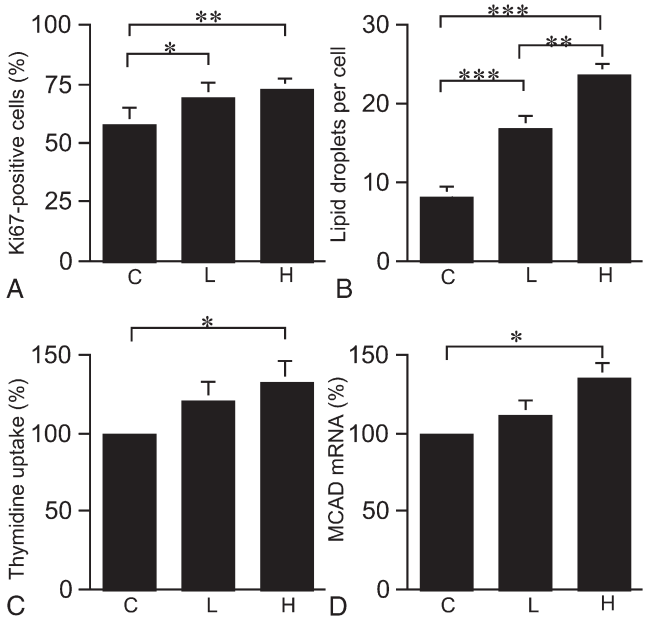

Fig. 4. MiaPaCa2 cells were incubated in control, low-FA, and high-FA media as described in the legend of Fig. 3. A, Ki67-positive cells were scored in 7 microscopic fields for each group. B, Lipid droplets in Fig. 3D to F were counted. C and D, Thymidine incorporation and MCAD mRNA were determined. Data in C and D came from 7 experiments. Each time, cells were incubated in triplicate in each medium. *P less than .05, **P less than .01, and ***P less than .001. C indicates control; L, low-FA medium; H, high-FA medium.

more positive cells and lipid droplets were found in cells with fatty acids (Fig. 4A, B). In addition, MiaPaCa2 cells incubated in the high–fatty acid medium showed increased thymidine incorporation and MCAD mRNA compared with the control values (Fig. 4C, D).

4. Discussion

Intracellular lipid droplets consist of a core of neutral lipids and a mantle of phospholipids. Normal cells in mice show increased lipid droplets when the animals are fed high-fat diet [4]. In vitro, exposure to fatty acids increases lipid droplets in breast cancer cells [14]. However, little is known about lipid droplets in cancer cells in vivo. In the present study, HF tumor cells contained numerous lipid droplets, suggesting that an increase in dietary fat increased intracellular lipid droplets in the tumor cells. In addition, HF tumors showed increased MCAD expression, suggesting a stimulation of the β-oxidation pathway by which fatty acids are metabolized as energy substrates. These data suggest that lipid metabolism was increased in the HF tumor cells. The increased lipid metabolism may be associated with an increased uptake of circulating fatty acids by the tumor cells. It may explain why HF increased plasma free fatty acids in HF-C but not HF-T mice. The increased lipid metabolism in HF tumor cells is consistent with our finding in vitro that exposure to fatty acids increased lipid droplets and MCAD mRNA in MiaPaCa2 cells.

Some previous studies evaluating the effects of high-fat diets on BOP-induced pancreatic cancer in hamsters used lard or corn oil as the source of dietary fat [7,8]. Both lard and corn oil are rich in saturated fatty acids, monounsaturated fatty acids, and n-6 polyunsaturated fatty acids (PUFAs) [15]. As a result, these diets increased the incidence of BOP-induced cancer compared with the cancer incidence seen in control animals with low-fat diets [7,8]. More recently, BOP-induced pancreatic cancer was studied in hamsters fed 3 different high-fat diets: a diet rich in n-3 PUFAs; a diet rich in n-6 PUFAs; and a diet containing a mixture of n-3, n-6, and n-9 PUFAs [16]. Although the incidence of pancreatic cancer did not differ between the 3 groups of BOP-treated animals, the hamsters on the diet rich in n-3 PUFAs had fewer macroscopically visible tumors [16]. Therefore, the fatty acid composition of high-fat diets may determine the effects of the diets on pancreatic cancer. In vitro, the survival of MiaPaCa2, Panc-1, and CFPAC human pancreatic cancer cells has been shown to be increased by saturated and monounsaturated fatty acids and decreased by PUFAs [11,12]. In the present study, the fat in the HF diet came from cocoa butter that was rich in saturated and monounsaturated fatty acids and in linoleic acid (an n-6 PUFA) [15]. To our knowledge, the present study is the first report on pancreatic cancer and high-fat diet using the orthotopic transplantation model. The HF tumors showed increased weight and central necrosis compared with the ND tumors. This suggests that increased dietary fat stimulated the turnover of MiaPaCa2 cells in vivo. In vitro, we found that thymidine incorporation in MiaPaCa2 cells was increased by oleic acid (monounsaturated) and linoleic acid.

How fatty acids regulate pancreatic cancer cell survival is unclear. Fatty acids have been found as energy substrates in prostate cancer cells [17]. In the current study, HF tumors had increased MCAD expression at both the protein and mRNA levels. This suggests that increased fatty acid oxidation was the mechanism by which HF stimulated the turnover of the tumor cells. Consistent with this view, fatty acid exposure increased mRNA for MCAD in MiaPaCa2 cells in vitro.

The increased cell turnover in HF tumors may also be mediated by ROS. Reactive oxygen species are stimulated by different factors including fatty acids [18,19]. On the one hand, ROS can activate signaling cascades toward cell proliferation [20]. On the other hand, ROS may subject cells to oxidative stress and cause cell death [20]. Thus, ROS can have dual effects on cell survival, inducing both cell growth and death. The dual effects of ROS have been seen in MiaPaCa2 and Panc-1 cells [21-25]. In the current study, SOD1 was measured as an index of ROS production. The HF tumors had increased SOD1, suggesting that ROS were involved in the increased cell turnover seen in the HF tumors.

Because the HF diet had 24% more energy than the ND, the increased cell turnover seen in HF tumors may have been a consequence of increased energy input. In the present study, many results (eg, MCAD expression and lipid droplet accumulation) are specific to lipid metabolism but not to a general increase in energy input. Therefore, the results seen in this study may be largely due to the increase in dietary fat rather than being due to a general increase in energy input. Our low- and high-fat diets also had differences in carbohydrate, protein, and fiber contents; and these differences could account for the results that we have obtained. However, the differences in the diet composition were less than those found in previous pancreatic cancer studies [26,27]. Thus, these differences may not have significant influences on the present results.

Pancreatic cancer is a leading cause of cancer-related death in Western countries, and diets in these countries are usually high in fat [28]. Further studies are needed to see whether the present results will also be seen when other human pancreatic cancer cell lines and other types of dietary fat are used in the orthotopic implantation model of pancreatic cancer. Such studies could help clarify the effects of high-fat diets on pancreatic cancer.

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