Promoting effect of arachidonic acid supplementation on N-methyl-N-nitrosourea-induced pancreatic acinar cell hyperplasia in young Lewis rats

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Abstract. Arachidonic acid (AA) is naturally found in human breast milk. AA, together with docosahexaenoic acid, is commonly added as a functional food ingredient to commercial infant formula worldwide, in accordance with the international standard of Codex Alimentarius. However, few studies have been performed that are concerned with the possible carcinogenic effects of AA supplementation during neonatal life. The effect of dietary AA supplementation in dams, during gestation and lactation, was investigated in N-methyl-N-nitrosourea (MNU)-induced preneoplastic lesions in the exocrine pancreas of young Lewis rats. Dams were fed either an AA (2.0% AA) or a basal (<0.01% AA) diet. On postnatal day 0 (at birth), male and female pups received a single intraperitoneal injection of either 35 mg/kg MNU or vehicle. The morphology and proliferating activity of the exocrine pancreas were examined by proliferative cell nuclear antigen immunohistochemistry 7, 14, 21, 28 and/or 60 days post-MNU. Histopathologically, acinar cell hyperplasia (ACH) occurred in the MNU-treated groups 60 days after MNU injection, irrespective of whether the rats had been fed an AA diet. Morphometrically, the number and area of ACH per 1 mm² in MNU-treated rats increased significantly in the AA diet-fed rats, compared with basal diet-fed rats. The number of proliferative cell nuclear antigen-positive acinar cells in both the normal and hyperplastic areas of MNU-treated rats increased significantly in the AA diet-fed rats. In conclusion, providing dams with an AA-rich diet during gestation and lactation promotes MNU-induced pancreatic ACH in young Lewis rats.

Introduction

Arachidonic acid (AA; 20:4n6) is a polyunsaturated fatty acid (PUFA) present in the phospholipids of cell membranes, and is particularly abundant in the retina and brain (1,2). AA present in the human body originates from dietary sources including egg yolk, or is synthesized from linoleic acid (3). AA is naturally occurring in human breast milk. AA, together with docosahexaenoic acid (DHA), is commonly added as a functional food ingredient to commercial infant formula worldwide, in accordance with the international standard of Codex Alimentarius (4). The consensus recommendations by the World Association of Perinatal Medicine and other such societies specify an intake of 0.2-0.5% DHA of total fatty acids and a level of AA at least equal to that of DHA (5). However, AA has been demonstrated to affect carcinogenesis in certain organs. Skin tumor models in mice, including the 12-O-tetradecanoylphorbol-13-acetate model, have demonstrated an essential role for the AA cascade in skin tumor promotion (6). AA has also promoted the growth of orthotopically transplanted breast cancer cell line tumors (KPL-1 cells) in female athymic BALB/c mice, and of urinary bladder tumors in a medium-term multi-organ rat carcinogenesis study (7).

Pancreatic cancer is the fourth leading cause of cancer-related mortality in the United States, with an estimated 42,470 new cases and 35,240 mortalities in 2009. This is due to a lack of early disease-specific symptoms and effective screening diagnostics, and poor prognosis at the time of diagnosis (8). Principle risk factors for pancreatic cancer include inherited germline mutations in genes and repeated exposure to N-nitroso compounds (through tobacco smoke and during the manufacturing of cured rubber products) or their N-nitrosamines precursors (in protein-containing foods dried at high temperatures) (9). Animal models of exocrine pancreatic carcinogenesis have been established in guinea pigs, and inducing experiments have elucidated the importance of the AA cascade in promoting carcinogenesis (10,11). Moreover, it has been reported that MNU is a powerful preneoplastic agent for inducing ACH in Lewis rats (12). In a medium-term multi-organ rat carcinogenesis study (7), ACH occurred in MNU-treated Lewis rats, and the incidence and multiplicity of ACH was significantly higher in MNU-treated Lewis rats (7). This study investigated the effect of dietary AA supplementation during gestation and lactation on pancreatic ACH in young Lewis rats.
University. Sixteen female SPF/VAF rats [LEW/CrlCrlj], 10-weeks-old and one-week pregnant, were purchased from Charles River Japan (Yokohama, Japan). Rats were maintained in specific pathogen-free conditions and had access to water and CE-2-modified diets containing different doses of AA ad libitum. Rats were housed in plastic cages with paper-chip bedding (Paper Clean, SLC, Hamamatsu, Japan) in an air-conditioned room at 22±2°C and 60±10% relative humidity, with a 12-h light/dark cycle. The illumination intensity inside the cages was less than 60 lux. Offspring were culled to a maximum of 10 per dam and the dams were maintained on their respective diets during the 21-day lactation period. During a post-weaning period of up to 60 days, the offspring were maintained on a CE-2 diet. A total of 116 male and female pups were used in this study. Five to ten rats were sacrificed at each time point, and there was a similar number of males and females in each dietary group.

**Chemical and dose formulation.** MNU was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was maintained at -80°C in the dark. MNU solution was dissolved in physiological saline containing 0.05% acetic acid immediately prior to use. MNU (35 mg/kg) or vehicle (physiological saline containing 0.05% acetic acid) was administered once by intraperitoneal (i.p.) injection. In our preliminary study, pancreatic ACH developed in 11 of the 14 surviving rats that were treated with 50 mg/kg MNU at birth (Fig. 1). However, almost 50% of these rats did not survive and all surviving female rats developed mammary cancers with severe hematotoxicity. Therefore, 35 mg/kg MNU was selected as a non-lethal, lower dose that would not cause mammary cancer to occur in the present short-term study.

**AA-supplemented diet.** AA (purity of 40.4% by our analysis) was purchased from Cargill Alking Bioengineering (Wuhan) Co, Ltd. (Hubei, China) and purified for use. The diet with 2.0 w/w% AA was semi-purified based on the modified CE-2 formulation (CLEA Japan, Inc., Tokyo, Japan). The basal diet consisted of modified CE-2. Gas chromatographic analyses of the fatty acid composition of the diets are displayed in Table I (18). The total fatty acid volumes were 47.20, 86.75 and 126.63 µg/mg of diet for the CE-2 (0.006% w/w AA), basal (0.008% w/w AA) and 2.0% AA diets, respectively. These diets were exposed to γ-ray (30 Gy) and formulated by CLEA Japan. Food was stored at 4°C to prevent lipid oxidation before use.

**Experimental procedures.** Male and female Lewis rats were exposed to either a basal or an experimental diet (2.0% AA) from fertilization to sacrifice. At birth (0 days of age), rats received an i.p. injection of either vehicle (physiological saline) or 35 mg/kg MNU (Fig. 2). At 7, 14, 21, 28 and 60 days after MNU or vehicle treatment, rats were anesthetized with isoflurane (Forane; Abbot Japan, Tokyo, Japan) and sacrificed by exsanguination from aortic transection. During the experiment, all pups were observed daily for clinical signs of toxicity, and were weighed at the time of MNU treatment and on the day of sacrifice. The pancreas was immediately removed on sacrifice and complete necropsies were conducted on all animals to check the systemic toxicities induced by
Table I. Fatty acid composition of diets given to rats.

<table>
<thead>
<tr>
<th>Fatty acid component (wt%)</th>
<th>CE-2</th>
<th>Basal</th>
<th>2.0% AA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>∑Saturated</td>
<td>19.53</td>
<td>23.06</td>
<td>24.45</td>
</tr>
<tr>
<td>∑Monounsaturated</td>
<td>23.65</td>
<td>34.55</td>
<td>24.41</td>
</tr>
<tr>
<td>∑n-6</td>
<td>41.81</td>
<td>31.82</td>
<td>42.26</td>
</tr>
<tr>
<td>∑n-3</td>
<td>7.20</td>
<td>5.53</td>
<td>3.86</td>
</tr>
<tr>
<td>n-3/n-6</td>
<td>0.17</td>
<td>0.17</td>
<td>0.09</td>
</tr>
<tr>
<td>AA/DHA</td>
<td>0.09</td>
<td>0.14</td>
<td>32.45</td>
</tr>
</tbody>
</table>

| n6                        |         |         |         |
| 18:2n6                    | 41.56   | 31.58   | 23.73   |
| 18:3n6                    | 0.00    | 0.00    | 0.85    |
| 20:2n6                    | 0.11    | 0.15    | 0.27    |
| 20:3n6                    | 0.00    | 0.00    | 1.40    |
| 20:4n6                    | 0.14    | 0.10    | 15.90   |
| 22:2n6                    | ND      | ND      | ND      |
| 22:4n6                    | ND      | ND      | 0.12    |
| 22:5n6                    | ND      | ND      | ND      |

| n3                        |         |         |         |
| 18:3n3                    | 3.16    | 3.51    | 2.29    |
| 20:3n3                    | 0.00    | 0.00    | 0.02    |
| 20:5n3                    | 2.04    | 1.07    | 0.91    |
| 22:5n3                    | 0.41    | 0.23    | 0.15    |
| 22:6n3                    | 1.59    | 0.73    | 0.49    |

| Total fatty acid (µg/mg)  | 47.20   | 86.75   | 126.63  |
| AA% of total fatty acid  | 0.30    | 0.12    | 12.56   |

AA, arachidonic acid; DHA, docosahexaenoic acid.

AA supplementation. Food consumption and body weight of the dams were measured once per week to estimate the true dosage of AA during the pregnancy and lactation periods.

Tissue fixation and processing. Pancreas tissues were fixed overnight in 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 4 µm and stained with hematoxylin and eosin (HE). Histopathological and morphometrical evaluations were performed by a toxicologic pathologist certified by the Japanese Society of Toxicologic Pathology and/or the International Academy of Toxicologic Pathology (K.Y. and A.T.). Previously defined histopathological terminology and diagnostic criteria were used. ACH is characterized by non-encapsulate glandular architecture with zymogen granules, exhibiting larger size cytoplasm and nucleus, basophilia, milder compression without higher mitotic index and nuclear polymorphism (19,20). This lesion was distinguished from regenerative hyperplasia that occurs in response to degeneration and necrosis of acinar cells, as regeneration in response to a single episode of severe toxic injury typically results in an almost complete restoration of normal architecture, rather than a focal nodular lesion (19). However, multifocal ACH develops into frank neoplasia (20).

Morphometric analysis of pancreatic ACH. HE-stained pancreas sections obtained from rats sacrificed 60 days after MNU treatment were scanned with a high-resolution digital slide scanner (NanoZoomer 2.0 Digital Pathology; Hamamatsu Photonics, Hamamatsu, Japan) to prepare digital images. The image files were opened with NDPview software (Hamamatsu Photonics). The total number of ACH lesions were counted on each digital image. The areas of each ACH and whole pancreas tissue were individually measured using NDPview software. To further evaluate the quantity and quality of ACH, the average area and number of ACHs per 1 mm² were calculated.

Proliferating cell nuclear antigen (PCNA) staining and labeling index. Formalin-fixed pancreatic sections from rats (basal and 2.0% AA diets) sacrificed 60 days after MNU or vehicle treatment were used for proliferative activity analysis. Sequential sections were immunohistochemically evaluated with anti-PCNA monoclonal antibody (clone PC10, dilution 1:100; Leica Biosystems, Newcastle-upon-Tyne, UK). A Labeled Streptavidin Biotin (LSAB) staining kit (Dako, Carpinteria, CA, USA) and antigen retrieval by pressure-cooker heating (Pascal, Dako) were used for immunostaining (21). PCNA-stained sections were scanned with a high-resolution digital slide scanner to prepare digital images. The labeling index was calculated from digital images by determining the number of PCNA-positive nuclei of acinar cells per 1 mm² in normal exocrine pancreatic tissue and ACH, using NDPview software.

Table I. Fatty acid composition of diets given to rats.
Statistical analysis. All discrete values, expressed as the mean ± standard error (SE), were analyzed using the two-tailed independent Student's t-test for unpaired samples, after confirming the homogeneity of variances. The incidence of ACH was analyzed using the \( \chi^2 \) test. The Results include comparisons between basal diet-fed rats and rats fed an AA-rich diet, in both the MNU-treated and vehicle-treated groups. P<0.05 was considered to indicate a statistically significant difference.

Results

General remarks. No fatalities occurred, and no clinical signs or symptoms related to treatment were evident in any of the pups or dams during the experimental period. None of the pups in any of the groups developed mammary tumors. The 2.0% AA diet did not influence food consumption in dams during the experimental period. During the pregnancy and lactation periods, the AA intake (mg/kg/day) of dams was 6.3 and 8.5 in the basal diet group, and 1,477 and 1,876 in the 2.0% AA group, respectively. The 2.0% AA diet did not influence body weight gain (the growth rate) in pups or cause weight changes in dams with or without MNU treatment. However, the growth rate of MNU-treated pups was typically lower than that of vehicle-treated pups (data not shown).

Morphological and morphometric analysis. The incidence of proliferative lesions of exocrine pancreatic tissue is demonstrated in Table II. In vehicle-treated rats with or without an AA-rich diet, no proliferative lesions were observed at any time point. In contrast, multifocal ACHs occurred in the basal diet-fed rats 60 days after MNU treatment (100% incidence). In rats fed an AA-rich diet, multifocal ACH occurred 28 and 60 days after MNU treatment (20 and 75% incidence, respectively). There was no significant difference in incidence between MNU-treated rats fed a basal diet and those fed an AA-rich diet. Regardless of diet, the majority of MNU-induced ACH were characterized by well-differentiated acidophilic acinar cells with cell polarity (Fig. 3). However, among MNU-treated rats, ACH were larger in rats fed an AA-rich diet, compared with rats fed a basal diet (Fig. 3). In the area surrounding the ACH, there was no evidence of capsulation. The neighboring pancreatic tissue maintained a normal acinar structure with or without mild compression. There was no significant difference in the incidence of ACH between males and females (data not shown). Endocrine pancreatic lesions and acinar cell tumors were not observed in any group at any time point.

The area and number of ACHs per 1 mm\(^2\) of exocrine pancreas were determined (Table III). In MNU-treated rats, the area of ACHs was 0.36±0.18 in rats fed a basal diet and 0.78±0.19 in rats fed an AA-rich diet; thus, the area of ACH was significantly greater in rats fed an AA-rich diet (\( P<0.01 \)). The number of ACHs increased significantly in MNU-treated rats fed an AA-rich diet (0.11), compared with rats fed a basal diet (0.08) (\( P<0.05 \); Table III). Thus, the number and area of ACH were highest in MNU-treated rats fed an AA-rich diet. This
result was consistent with the morphological characterization (Fig. 3).

**Macrosopic examination of other organs.** Atrophic changes in the spleen, thymus and testis occurred 7-28 days after MNU treatment, regardless of diet. No macroscopic lesions were detected in any organs, including the pancreas, 60 days after MNU treatment.

**PCNA index.** In order to compare the cell kinetics of normal acinar areas and ACH areas among rats fed a basal or AA-rich diet, the PCNA-positive cell number per 1 mm² of exocrine pancreas was determined. Changes in the number of PCNA-positive acinar cells 60 days after MNU treatment are displayed in Fig. 4. In the basal and AA-rich diet groups, PCNA-positive cells gradually and significantly increased in the normal areas of vehicle- and MNU-treated rat exocrine pancreas, and in the MNU-treated hyperplastic areas. In the normal areas of vehicle-treated rats fed a basal diet or AA-rich diet, the numbers of proliferative acinar cells were 133.2 and 121.0 (no significant difference). In contrast, the number of PCNA-positive cells in normal areas increased significantly in MNU-treated rats fed an AA-rich diet (359.7), compared with rats fed a basal diet (298.7) (Fig. 4A). The number of PCNA-positive cells in ACHs was significantly increased in MNU-treated rats fed an AA-rich diet (786.6), compared with rats fed a basal diet (662.7) (Fig. 4A). Thus, the proliferative activity of acinar cells in normal and ACH areas was highest in MNU-treated rats fed an AA-rich diet (Fig. 4B).

**Discussion**

The main purpose of the present study was to determine whether increased levels of AA during gestation and lactation proportionally enhanced the development of preneoplastic ACHs in the pancreas of MNU-treated rat pups. Pancreatic morphology in rats treated with 35 mg/kg MNU exhibited small ACH in rats fed a basal diet and large ACH in rats fed an AA-rich diet (2.0% AA). The number and area of ACHs increased in MNU-treated rats fed an AA-rich diet, which was consistent with the morphological characterization. By PCNA immunohistochemistry, the proliferative activity of acinar cells in both the normal and hyperplastic areas was demonstrated to have significantly increased in MNU-treated rats fed an AA-rich diet. These results demonstrated that 2.0% AA had a strong promoting effect on ACHs, both morphologically and morphometrically. In the MNU rat model of pancreatic carcinogenesis with exposure to 50 mg/kg MNU at 3 days of age, mancozeb, diethyldithiocarbamate, bayleton, phenobarbital and folinithion enhance preneoplastic lesions (acinar cell hyperplasia and dysplasia), carcinoma in situ and/or adenocarcinoma at 24 weeks of age (16,17,22). In our preliminary study, 50 mg/kg MNU induced mortality and mammary cancers with severe hematotoxicity. Lewis rats are more sensitive than other rat strains to chemical carcinogenesis in the exocrine pancreas. Additionally, rats are most sensitive to the induction of tumors if the chemical is administered during the first several weeks after birth, when the rate of cell division in the pancreas is highest (19). Therefore, as with our experimental protocol, a short-term study (60 days) with 35 mg/kg MNU as a non-lethal, lower dose that does not cause mammary cancer occurrence, may be extremely useful for testing the promoting, progressing or inhibitory effect of chemical and physical agents on cell proliferation and transformation of rat exocrine pancreas.

High levels of dietary PUFA promote tumor growth in several animal models, including pancreatic cancer models (23). A higher incidence of proliferative exocrine lesions in the pancreas have been observed in F344 rats given
corn oil in long-term studies (19,24). The promoting effects of unsaturated fats have been attributed to the development of these spontaneously initiated lesions (19,20). In corn oil-treated models, males have a higher incidence and wider distribution of ACH and tumors than female rats (25), and testosterone is considered to be responsible for the higher incidence of these lesions in males. In the present study, sex differences in the incidence of MNU-induced ACH were not evident (data not shown), which is likely due to the shorter study period (60 days).

Linoleic acid (LA; 18:2n6) is partly responsible for the promoting effect of dietary polyunsaturated fats on pancreatic carcinogenesis via accelerated prostaglandin synthesis caused by the metabolism of linoleic-derived AA in preneoplastic tissue (23,26). The strongest enhancing effect on the growth of pancreatic (pre)neoplastic lesions in the azaserine rat model and N-nitroso bis(2-oxopropyl)amine (BOP) hamster model was obtained with LA-rich diets. In azaserine-induced ACH (atypical acinar cell foci) in rats fed LA-rich diets, the BrdU-labeling index was significantly increased, compared with rats fed LA-low diets (26). In our study, the proliferative activity of acinar cells in normal and ACH areas was highest in the MNU-treated rats fed an AA-rich diet, compared with those fed a basal diet. The LA concentration of the AA-rich diet in our study was lower than that of the basal diet (Table I); therefore, LA was not responsible for the promoting effect on ACH in the present study.

Cholecystokinin (CCK), which is produced and secreted by highly specialized enteroendocrine cells located in the duodenal and jejunal mucosa of the gut, strongly stimulates the secretion of amylase from acinar cells (19,27). Feeding raw soybean flour to rats produces hyperplasia and neoplasia of the exocrine pancreas (28). Additionally, prolonged injection of CCK accelerates the production of atypical acinar cell foci and invasive cancers (11,19,20). Pancreatic growth is considered to be stimulated by interference in the feedback control of CCK by a heat-labile soybean trypsin inhibitor. This effect appears to be independent of any promotion by the high levels of unsaturated fats in the raw soybean flour (11,20,29). The composition of dietary fatty acids influences the CCK secretory response. Short-term exposure of enteroendocrine STC-1 cells to AA promotes CCK secretion (30). Persistent stimulation of the pancreas by CCK induces acinar cell hypersecretion of amylase, followed by hyperplasia, which is capable of promoting chemically induced carcinogenesis of the exocrine pancreas (27,29). Exogenous AA induces amylase secretion in a concentration-dependent manner in the rat acinar cell ex vivo model, suggesting a role for AA as a potential intracellular mediator in the exocrine pancreas (31). In the present study, this information collectively supports our speculation that CCK-related amylase release is involved in the promoting effects of AA on MNU-induced ACH.

Pancreatic cancer is the fourth leading cause of cancer mortality in the United States (8). A previous large population-based, case-control clinical study in San Francisco Bay provided evidence that the saturated fatty acids, monounsaturated palmitoleic and oleic fatty acids, and polyunsaturated LA may increase the risk of adenocarcinoma of the exocrine pancreas, whereas gadoleic acid (monounsaturated) and ω-3 fatty acids (polyunsaturated) may decrease this risk (8). However, no association was observed between pancreatic cancer risk and a dietary intake of 160 mg or more of AA. AA supplementation by healthy adults appears to confer no toxicity or significant safety risk; daily dosages of 1,500 mg for 50 days in the United States and 838 mg for 14 days in Japan have been well-tolerated in clinical studies with no significant side effects (32,33). Previously, AA demonstrated no promoting effects on a rat medium-term multi-organ carcinogenesis model using five carcinogens including MNU (34). The recommended intake of AA in Japan is 24 mg/kg/day in adult humans (http://www.suntory-kenko.com/supplement/main/433461; in Japanese). The 2.0% AA diets used in the present study provide an AA dose of 1,477 mg/kg during pregnancy and 1,876 mg/kg during lactation, which are 61.6- and 78.2-fold higher than the recommended human dose, respectively. Moreover, daily AA intake by Japanese infants via breast milk is approximately 14.3 mg AA/kg/day (34). Compared with the amounts of AA tested in the present study, this is approximately 103- and 131-fold higher. Taken together, an AA-enriched diet in the prenatal and postnatal periods is not likely to cause exocrine pancreatic carcinogenesis in humans.

In conclusion, an AA-rich diet in dams during gestation and lactation promotes MNU-induced pancreatic ACH in young rats. An AA-rich diet induces increased proliferative activity of acinar cells following MNU initiation, likely followed by the development of exocrine pancreatic tumors. Several factors, including AA itself, may affect the increased proliferative activity of the exocrine pancreas. Further studies of the cascade of proliferative action are necessary to understand the detailed mechanisms of the promoting effects of AA on exocrine pancreatic carcinogenesis.

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