EFFECT OF DIETARY N-3 AND N-6 POLYUNSATURATED FATTY ACIDS ON LIVER METASTASIS OF HUMAN COLONIC ADENOCARCINOMAS IN NUDE MICE

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Abstract: Nude mice at 4-6 weeks of age were fed diets containing 10 % oil with different α-linolenic acid (α-LnA, n-3)/linoleic acid (LA, n-6) ratios; Group A (n-3/n-6 =0.01), Group B (0.30), Group C (0.87) and Group D (3.37). After 10 days, each dietary group of mice received intrasplenic (i.s.) transplantation of human colonic adenocarcinomas and was fed the same diet. Animals were sacrificed at 3 weeks after i.s. transplantation of COL 5 with a higher metastatic potential and at 2 months in the case of COL 4 with a lower metastatic potential. The incidence of liver metastasis and the average number of metastatic nodules per mouce decreased in the order of Groups B>C>D, A. Dietary α-LnA (n-3)/LA (n-6) ratio was reflected mainly in eicosapentaenoate (EPA, n-3)/arachidonate (AA, n-6) ratio in the phospholipid fractions and mainly in α-LnA (n-3)/LA (n-6) ratio in the triacylglycerol fractions from both the non-cancerous reference tissues and the metastatic tumor tissues in liver, similarly. The thromboxane (TX) A_2 and prostaglandin (PG) I_2 levels decreased along with the increase in EPA/AA ratio of phospholipids, but the TXA_2/PGI_2 ratio was the highest and the bleeding time was the shortest in Group B as compared with the other dietary groups. Thus, the metastatic potential of intrasplenically transplanted human colonic carcinoma cells was positively correlated with both the TXA_2/PGI_2 ratio in liver and thrombotic tendency, but not with dietary α-LnA/LA ratios in nude mice.

Index Terms

n-3/n-6 ratio, human colonic adenocarcinoma, intrasplenic transplantation, nude mouse, TXA_2/PGI_2 ratio

INTRODUCTION

α-linolenic acid (α-LnA, n-3) and linoleic acid (LA, n-6) are synthesized in plants but not in animal tissues. When ingested, α-LnA is desaturated and elongated to form n-3 fatty acids such as eicosapentaenoic acid (EPA, n-3) and docosahexaenoic acid (DHA, n-3) while LA is converted to arachidonic acid (AA, n-6); no inter-conversion occurs between the n-3 and the n-6 series of fatty acids in animal bodies. Various foods contain different proportions of n-3 and n-6 fatty acids, and the n-3/n-6 ratio of tissue lipids is known to be affected by the choice of foods. Starting from epidemiological studies on Greenlanders, n-3 fatty acids have been proved to be beneficial for the prevention of ischemic heart diseases[1,2,3]. The n-3/n-6 ratio has also been shown to affect blood pressures[4], learning behavior[5,6], allergic reactivity[7] and many other physiological aspects[8]. Carcinogenesis of most western type cancers (mammary, colon,

prostate, pancreas, lung, esophagus and others) has been shown to be promoted by dietary n-6 fatty acid (linoleic acid) and suppressed by n-3 fatty acids[9]. Because there are certain limitations in converting tumor cells into normal cells, regulation of tumor cell metastasis has attracted attention as a promising target in cancer therapy. The successful metastasis of the tumor cell consists of the following steps: 1) detachment of tumor cells from primary neoplasm; 2) penetration of blood vessels or lymphatics passive dissemination to distant sites; 3) lodgement and adhesion to blood vessels in host organs; and 4) extravasation and subsequent survival and proliferation to form new tumor foci. Spontaneous metastasis involves steps 1) ~4) and experimental metastasis involves steps 3)~4). In animal models, diets rich in linoleic acid (n-6) have been shown to stimulate, and those rich in n-3 fatty acids to suppress, metastasis of tumor cells[10-14].

Hubbard et al[14] reported that high levels of dietary linoleic acid increased the spontaneous metastasis of mouse mammary tumors by influencing the lodgement, implantation and survival, but not the proliferation, of fumors. The lodgement and adhesion to blood vessels of tumor cells are influenced by platelet aggregability of the host, which is known to be affected by dietary n-3 and n-6 polyunsaturated fatty acids (PUFAs). Prostaglandins (PG) I_2 and I_3 derived from AA (n-6) and EPA (n-3), respectively, have comparable anti-aggregatory activities while pro-aggregatory activity of thromboxane (TX) A_2 derived from AA is much higher than that of TX A_3 derived from EPA[48,51]. Furthermore, n-3 fatty acids, particularly EPA, competitively inhibit AA metabolism, and these mechanisms appear to form the major bases for the decreased platelet aggregability induced by dietary n-3 fatty acids[16-19].

Intrasplenic (i.s.) injection of human carcinoma suspension is a good model for liver metastasis[20-23]. In this paper, the effect of dietary α-linolenate (n-3)/linoleate (n-6) ratio (α-LnA/LA ratio) on experimental liver metastasis was examined using the method of i.s. transplantation of human colonic adenocarcinomas. Bleeding time and eicosanoid ratio were also determined as parameters associated with dietary n-3/n-6 ratio.

MATERIALS AND METHODS

Animals and diets

Male BALB/c AJcl-nu nude mice at 4-6 weeks of age were obtained from Nihon Clea Co., Tokyo. Mice divided randomly into 4 groups (Groups A-D) were fed diets supplemented with perilla seed oil or safflower seed oil or mixtures of these oils. Sterilized distilled water was given ad libitum in CLEATRON (Nihon Clea Co.), which was specially designed for the maintenance of nude mice. Diets were prepared by extracting a conventional laboratory diet (Nihon Clea Co., CE-2) with n-hexane and then supplementing the defatted diets with a vitamin mixture (2 % (w/w)) (Nihon Clea Co.) and oils (10 w/w %). The α-linolenate/linoleate ratios of the diets were 0.01 in Group A, 0.30 in Group B, 0.87 in Group C. and 3.37 in Group D (Table 1). The diets were stored at 4 ℃ and those provided were replaced every other day to maintain the peroxide values of diets below 30 meq/kg.

Human colonic cancers for transplantation

Table 1. Fatty acid compositions of diets

Fatty acid	GroupA	GroupB	GroupC	GroupD
Myristic, 14 : 0	0.2	0.2	0.6	2.5
Palmitic, 16 : 0	7.5	7.7	7.2	9.8
Palmitoleic, 16 : 1	0.2	0.3	0	0
Stearic, 18 : 0	2.6	2.5	2.3	2.5
Oleic, 18 : 1 n-9	13.8	14.1	17.4	18.5
α-Linolenic, 18 : 3 n-3 (α-LnA)	0.7	15.9	33.8	50.9
Linoleic, 18 : 2 n-6 (LA)	74.6	52.8	38.7	15.1
Gondoic, 20 : 1	0.4	0.4	0.3	0
α-LnA/LA ratio	0.01	0.30	0.87	3.37

(% of total fatty acids)

Human colonic cancers resected from a 58-year-old man (COL 4) and a 40-year-old woman (COL 5), were obtained from the Central Institute for Experimental Animals (Tokyo). The histopathological type of these cancer cells was well differentiated adenocarcinoma. COL 4 transplanted via i.s. into nude mice has a lower liver metastatic potential than COL 5[24].

Experimental designs

The bleeding time from the tail vein was measured after feeding the respective diets for 10 days, four nude mice from each dietary group being selected at random and sacrificed for the measurements of liver TXA_2 and PGI_2 levels after the measurement of bleeding time (Experiment 1). The other nude mice received intrasplenic (i.s.) injection of tumor cell suspensions prepared by trypsinization of the subcutaneously grown COL 4 and COL 5 tumors and were fed the same diets continuously. The incidence of liver metastasis and macroscopic measurement of liver nodules/mouse as well as the fatty acid compositions of lipids from non-cancerous liver tissues (reference tissues) and metastatic tumors were determined (Experiment 2).

Measurement of bleeding time

After feeding the respective diets for 10 days, tail roots of nude mice were fixed to a plate by taping and the tail tip (8 mm long) was cut off with a klife. The blood from the peripheral tail vein was absorbed with a piece of paper every 30 sec. until bleeding from the tail vein stopped[25].

Analysis of liver tissue TXA_2 and PGI_2 levels

After feeding the diets for 10 days, the middle lobe of liver was removed from a mouse, some cut lines were given with a knife, rinsed lightly by shaking in an indomethasin solution (0.1 mM indometasin-10 mM EDTA in 7 ml saline) kept in ice, and rapidly stored in liquid nitrogen. The frozen liver tissues were mixed with 95 % ethanol and homogenized in tubes kept on dry ice[26,27]. TXA_2 and PGI_2 concentrations (pg/ml) were measured by radioimmunoassay, using rabbit antisera against stable metabolites of TXA_2 and PGI_2 (anti-TXB_2 and anti-6-keto-$PGF_{1\alpha}$,

Radioimmunoassay kit [1951] in NEN Corporation)[28,29]. Total proteins in the precipitates were measured by Lowry's method[30].

Intrasplenic injection of tumor cell suspension

The seventh passage subcutaneous tumors were minced in Eagle's minimum essential medium (MEM) containing an antibiotic (gentamicin, 50 μg/ml). The tumor pieces were then digested with 0.25 % trypsin in Eagle's MEM for 15 miniutes at 37 ℃. After passage through a cytosieve (100 mesh/cm^2), the cell suspension was rinsed twice with Eagle's MEM. Cell viability was determined by the trypan blue exclusion method. Mice were anesthetized with diethylether. A small incision was made in the left flank through the skin and peritoneum, and the spleen was carefully exposed. Viable tumor cells (1.0×10^6 cells) in 0.2 ml Eagle's MEM were then injected into the lower pole of the spleen using a 21-gauge needle. After slightly compressing the spleen with cotton gauze for one minute to stop splenic bleeding, splenectomy was done and then the abdominal wall was closed with sutures[23].

Fatty acid analysis of non-cancerous reference tissues and metastatic tumors

At the end of feeding the test diets in Experiment 1, a piece of non-cancerous reference tissue and metastatic tumor (more than 200 mg each) were enucleated from the livers of mice and stored at -80 ℃ until analysis. The total lipids of the isolated liver were extracted with chloroform/methanol according to the method of Bligh and Dyer[31], and the phospholipids and triacylglycerols were separated by one-dimensional thin-layer chromatography on Silical Gel (Merck 60) using petroleum ether/diethyl ether/acetic acid (80 : 30 : 1, v/v/v) as a solvent. Fractions were located by spraying with ethanolic Rhodamin 6 G. Phospholipids were recovered from the adsorbent by extracting with chloroform/methanol/6 %-ammonium hydroxide (6 : 5 : 1, v/v/v) and triacylglycerols with chloroform/methanol (2 : 1, v/v). The fatty acids in the phospholipids and triacylglycerols were transmethylated with 5 % HCI/MeOH. Fatty acid methyl esters were then quantitated by gas-liquid chromatography with a capillary column using heptadecanoic acid as an internal standard.

Statistical analysis

All data were analyzed by unpaired Student's t-test.

RESULTS

Effect of dietary α-LnA/LA ratio on liver metastatic potential

The gain rates of body weight and liver weight/gain showed no significant differences among the dietary groups of mice transplanted with COL 4 and COL 5 (data not shown). The incidences of liver metastasis and liver metastatic nodules/mouse in COL 4 and COL 5 are shown in Table 2. The incidence of liver metastasis in COL 4 was significantly higher in Group

B (85.7 %) than in Groups A and D (28.6 %). The incidence of liver metastasis of COL 5 was 100 % in all the dietary groups. Liver metastatic nodules/mouse was also the highest in Group B as compared with Group A and Group D in both COL 4 and COL 5. The metastatic potential

Table 2. Effect of dietary oils on liver metastatic potential of human colonic adenocarcinomas

Dietary group (α-LnA/LA)	No. of mice with liver nodules /No. of mice attempted(%)		Mean No.±S. D. of liver nodules/mouse	
	COL4	COL5	COL4	COL5
A(0.01)	2/7(28.6)[a]	7/7(100)	1.1±2.3[a]	48.9±31.3[c]
B(0.30)	6/7(85.7)[a,d]	7/7(100)	8.1±6.0[a,b]	125.7±40.4[c,d]
C(0.87)	4/7(57.1)	7/7(100)	5.1±8.4	92.0±47.5
D(3.37)	2/7(28.6)[d]	7/7(100)	0.7±1.3[b]	68.4±35.1[d]

a : significant difference between A and B ($p<0.05$)
b : significant difference between B and D ($p<0.01$)
c : significant difference between A and B ($p<0.01$)
d : significant difference between B and D ($p<0.05$)

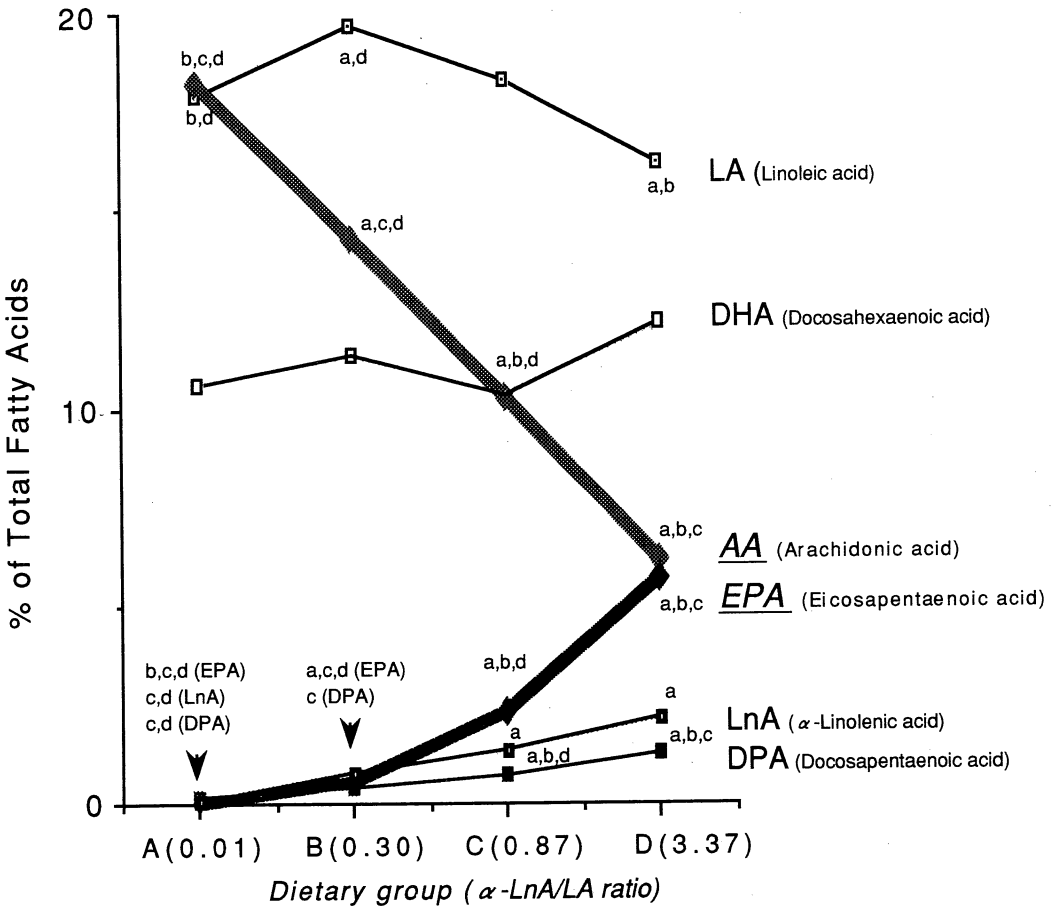

Fig. 1-a. Fatty acid compositions of phospholipids in non-cancerous reference tissues in liver.
a ; 〈A〉 : 〈others〉 b ; 〈B〉 : 〈others〉 c ; 〈C〉 : 〈others〉 d ; 〈D〉 : 〈others〉
Values are expressed as means. (n=3 unless otherwise indicated)
Statistical significance was analyzed by an unpaired Student's t-test. (a, b, c, d, $p<0.05$)

tended to decrease when the dietary α-LnA/LA ratio was increased from 0.30 to 3.37, but Group A with a dietary α-LnA/LA ratio of 0.01 did not fit into this generalization.

Fatty acid compositions from non-cancerous reference tissues and metastatic tumors

Fatty acid compositions of tissue phospholipids were affected significantly by the diets (Fig. 1-a, Fig. 1-b). The dietary α-LnA (n-3)/LA (n-6) ratio was reflected mainly in the proportions of EPA (n-3) and AA (n-6) ; the proportions of α-LnA and LA were affected to a relatively lesser degree. These differences were essentially the same both in the reference tissues and in the metastatic tumors (COL 4). In the triacylglycerol fractions, however, major differences were seen in the proportions of α-LnA and LA but the proportions of EPA and AA were affected less by the diets (Fig. 2-a, Fig. 2-b). Again, no significant difference was seen in the fatty acid compositions of triacylglycerols from the reference tissues and the metastatic

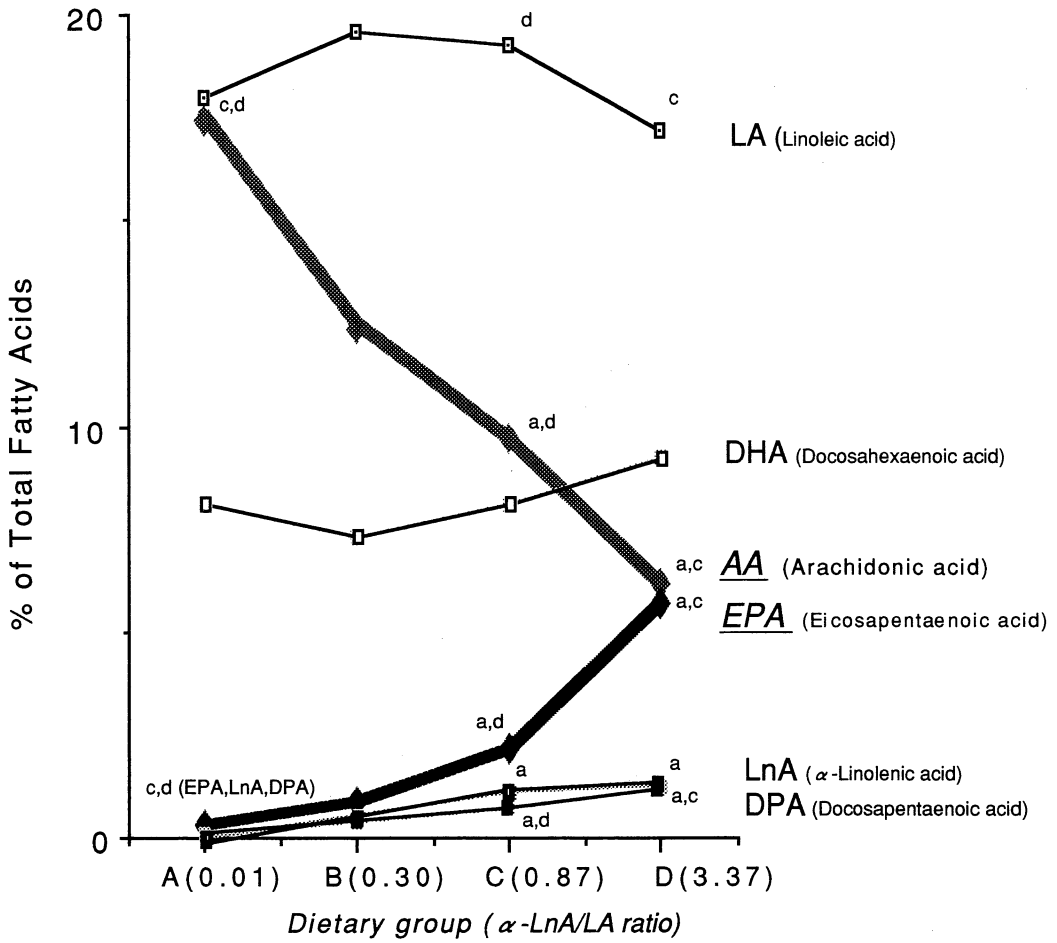

Fig. 1-b. Fatty acid compositions of phospholipids in metastatic tumors (COL4) in liver.
a ; 〈A〉 : 〈others〉 b ; 〈B〉 : 〈others〉 c ; 〈C〉 : 〈others〉 d ; 〈D〉 : 〈others〉
Values are expressed as means. (n=3 unless otherwise indicated, n=1 in group B)
Statistical significance was analyzed by an unpaired Student's t-test. (a, b, c, d : $p<0.05$)

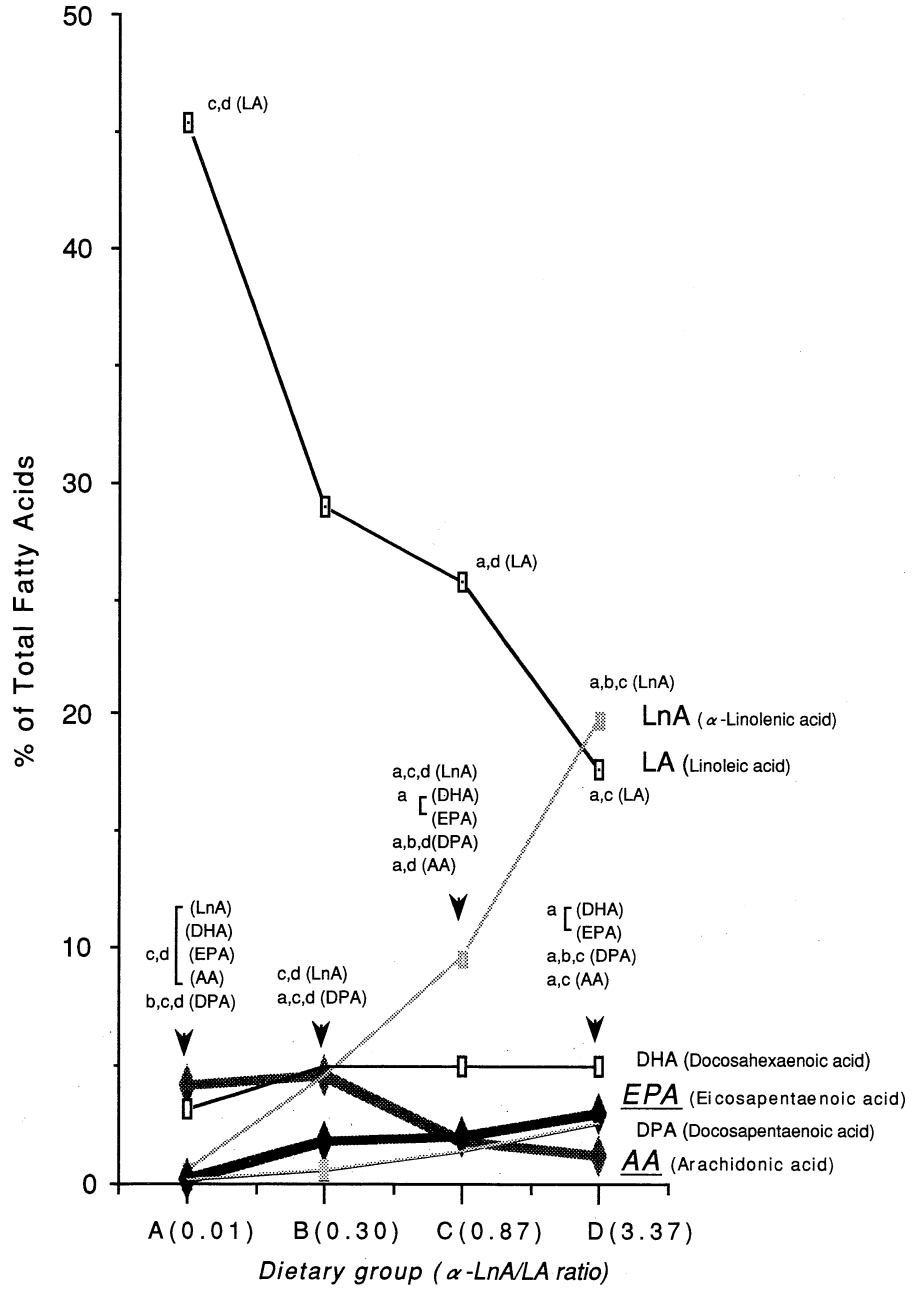

Fig. 2-a. Fatty acid compositions of triacylglycerols in non-cancerous reference tissues in liver.
a ; 〈A〉 : 〈others〉 b ; 〈B〉 : 〈others〉 c ; 〈C〉 : 〈others〉 d ; 〈D〉 : 〈others〉
Values are expressed as means. (n=3 unless otherwise indicated)
Statistical significance was analyzed by an unpaired Student's t-test. (a, b, c, d : $p < 0.05$)

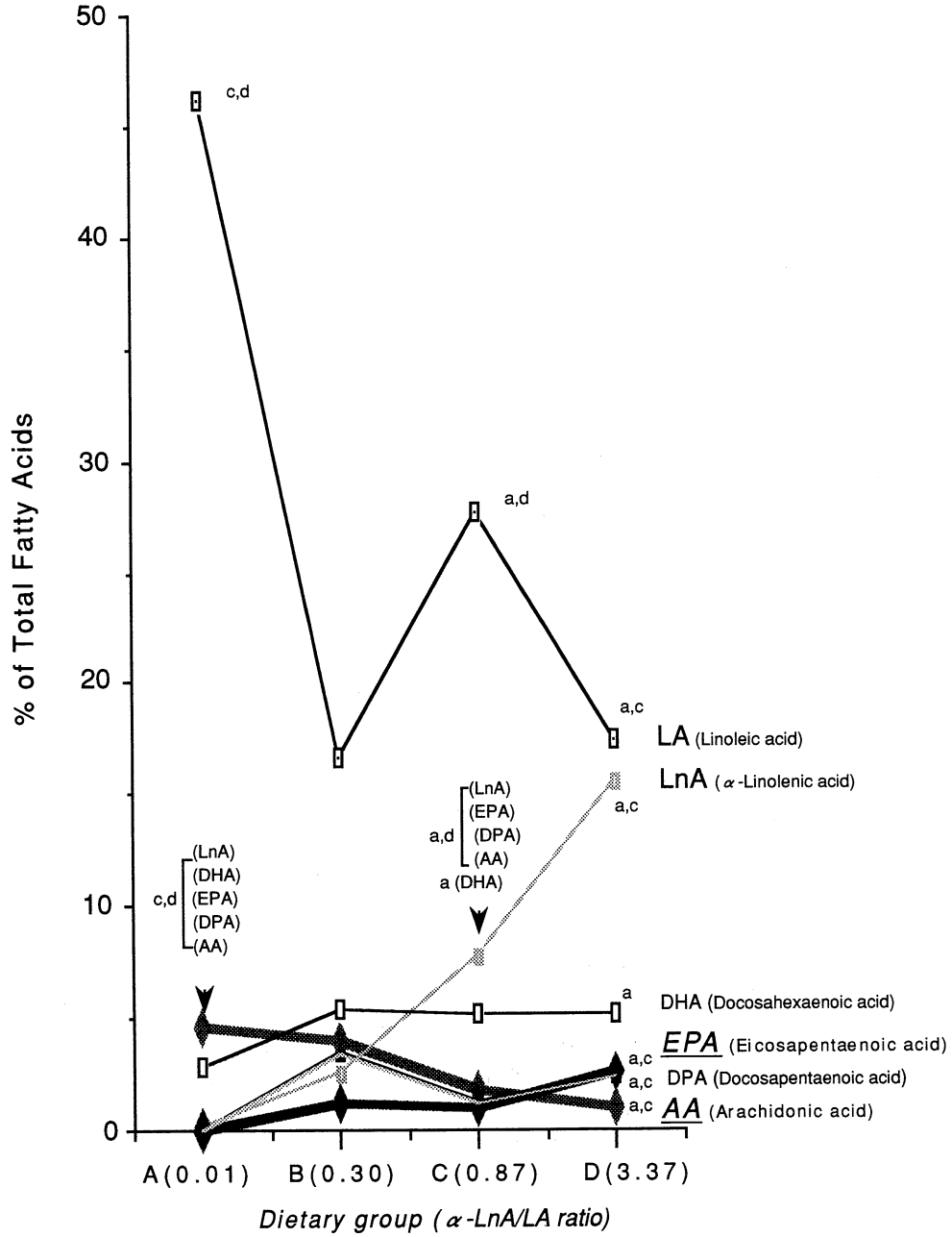

Fig. 2-b. Fatty acid compositions of triacylglycerols in metastatic tumors (COL4) in liver.
a ; 〈A〉 : 〈others〉 b ; 〈B〉 : 〈others〉 c ; 〈C〉 : 〈others〉 d ; 〈D〉 : 〈others〉
Values are expressed as means. (n=3 unless otherwise indicated, n=1 in group B)
Statistical significance was analyzed by an unpaired Student's t-test. (a, b, c, d : $p<0.05$)

tumors. The proportion of DHA was not affected by the dietary α-LnA/LA ratio.

Eicosanoid levels in liver and bleening time

Table 3. Eicosanoid levels in liver and bleeding time

Dietary group	TXA_2 measured as TXB_2 (pg/mg protein) (n=4)	PGI_2 measured as 6-keto-$PGF_{1\alpha}$ (pg/mg protein) (n=4)	TXA_2/PGI_2 ratio	Bleeding time (minutes) (n=11)
A	5.18 ± 0.84^{a}	3.44 ± 0.47^{c}	1.51 ± 0.15^{d}	12.61 ± 3.51^{f}
B	4.73 ± 1.16^{b}	2.26 ± 0.43^{c}	$2.07\pm0.19^{d,e}$	$8.61\pm4.62^{f,g}$
C	3.01 ± 0.75^{a}	1.78 ± 0.28^{c}	1.68 ± 0.22^{e}	11.11 ± 3.01^{h}
D	$2.87\pm0.37^{a,b}$	1.63 ± 0.30^{c}	1.68 ± 0.10^{e}	$15.21\pm2.71^{g,h}$

a : significant difference between A and C, D ($p<0.05$)
b : significant difference between B and D ($p<0.05$)
c : significant difference between A and B, C, D ($p<0.01$)
d : significant difference between A and B ($p<0.01$)
e : significant difference between B and C, D ($p<0.05$)
f : significant difference between A and B ($p<0.05$)
g : significant difference between B and D ($p<0.01$)
h : significant difference between C and D ($p<0.05$)

The contents of 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2) and TXB_2 (stable metabolite of TXA_2) were determined in the livers from control mice without transplantation of colonic adenocarcinomas. Both the TXA_2 and PGI_2 levels decreased along with the increase in EPA /AA ratio of tissue phospholipids (Table 3). However, the TXA_2/PGI_2 ratio was higher and the bleeding time was shorter in Group B than in the other dietary groups (Table 3).

DISCUSSION

The platelet may play an important role in metastasis by protecting malignant cells in the blood stream[32]. The association of circulating tumor cells with host platelets has been suggested from the findings that anticoagulants such as heparin[33], warfarin[34], asprin[35], indomethasin[36], dypridamole[37], PGI_2[38,45] and inhibitors of TXA_2 synthesis[38-40,44] were inhibitory, but coagulants such as ADP[41], anti-PGI_2 inhibitor (15-HPETE)[38] and TXA_2 were stimulatory for the metastasis of tumor cells. Both PGI_2 and TXA_2 exert antagonistic effects on platelet aggregation by opposing effects on platelet cyclic AMP (cAMP) ; TXA_2 prevents increase in platelet cAMP in response to external stimuli[42], but PGI_2 inhibits aggregation by increasing platelet cAMP level[43]. Therefore, TXA_2/PGI_2 ratio can be an indicator of metastatic potential as well as of platelet aggregability.

It has been shown that tumor cell metastasis parallels roughly the TXA_2/PGI_2 ratio and bleeding tendency, which were the highest in Group B with a dietary α-LnA/LA ratio of 0.03, but does not parallel the AA/EPA ratio of tissue phospholipids (Table 2, Table 3, Fig. 1-a and Fig. 1-b). In the present model, liver metastatis of human colonic adenocarcinomas in nude mice was not significantly different between the safflower oil group (Group A) and the perilla oil group (Group D). This is apparently inconsistent with earlier observations by Watanabe et al.[49] and by Hori et al.[10] that perilla seed oil rich in α-LnA (n-3) inhibits platelet aggregability and suppresses the metastasis of hepatoma cells to the lung in rats. The reason for this apparent discrepancy is not clear but may be related to the unique immunological system in nude mice. Nevertheless, the present experiments have revealed that dietary oils with extremely low n-3/n-6 ratios (e.g., safflower oil) do not always stimulate metastasis in animal models. The n-3/n-6 fatty acid ratio of liver phospholipids roughly paralleled the dietary α-LnA/LA

ratio but not the TXA_2/PGI_3 ratio. The PGI_3 and TXA_3 derived from EPA, which were not determined in the present experiments, must also be considered because their physiological activities and the productivities are different from those of eicosanoids derived from AA[48,50-51]. Other unidentified factors such as the levels of enzymes involved in eicosanoid synthesis may also be involved in the dietary effects of n-3 and n-6 fatty acids on tumor cell metastasis.

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