Physicochemical Perturbation of α-Linolenic Acid Related to Cell Proliferation

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The influence of fatty acids, α-linolenic acid (ALA), and linoleic acid (LA), on lipid membrane dynamics and cell proliferation was investigated. Electron-spin resonance measurements with 2-hexyl-2-(10-methoxycarbonyldecyl)-4,4-dimethyl-3-oxazolidinyloxyl revealed that the penetration of ALA or LA into the liposomal membrane causes an increase in the membrane fluidity and a decrease in the phase-transition temperature of the lipid membrane. When human colon cancer cells (RPMI4788 and BM314) and normal mouse fibroblast (L-cell) were incubated with ALA, the membrane fluidity of the cancer cells significantly increased over that of normal cells. ALA made the cancer cell membrane more fluid than did LA. When ALA was administered at a concentration of 40 $\mu g\,ml^{-1}$, it showed a strong cytotoxicity against the cancer cells, but not to the normal cells. On the other hand, LA (up to 80 $\mu g\,ml^{-1}$) rather promoted growth of the cancer cells. These results seem able to be interpreted as an aspect of the selective cytotoxicities of ALA.

The influence of fatty acids on cell-membrane dynamics has been extensively investigated from various aspects. The fatty acid moiety of membrane lipids can be replaced without any serious membrane damage by changing the lipid composition in the feed to animals or in the cell culture medium.[1,2] The replacement occasionally affects the membrane fluidity.[3—6] Furthermore, the membrane-fluidity change induced by the fatty acids influences the cell function regarding various aspects, such as the transport of an antineoplastic drug,[1] the activity of a membrane-bound enzyme,[7] endocytosis,[8] and amino acid transport.[9] Those may lead to a depression of cell differentiation and/or proliferation because of biological or physicochemical damage to the cell membrane. A sort of polyunsaturated fatty acids (PUFAs), ω-3 type fatty acids (PUFAs having a double bond on the third carbon atom from the terminal methyl group (at the ω-position)) such as α-linolenic acid, icosapentaenoic acid, and docosahexaenoic acid, inhibits in vivo tumor growth,[10—12] while linoleic acid (ω-6 type fatty acids PUFAs having a double bond on the sixth carbon atom from the terminal methyl group (at the ω-position)) enhances the growth rate of transplanted mammary adenocarcinoma.[13,14] The metabolites of PUFAs, such as lipid peroxide[15—17] or prostaglandin,[18—23] are also related to cell viability. In addition, any alteration or modification of the membranous lipids of hepatoma cells cultured with PUFAs increases the susceptibility of the cell to complement-mediated cytolysis[24] and natural killer cells.[25]

We would like to report here on the physicochemical perturbation of 9,12,15-octadecatrienoic acid (α-linolenic acid, ALA) and 9,12-octadecadienoic acid (linoleic acid, LA) on the cell membrane fluidity of mouse fibroblast (L-cell) and human colon cancer cells (RPMI4788 and BM314). In order to understand the relationship between the cell membrane fluidity and the selective cytotoxicity of ALA to tumor cells,[26—28] the effect of the supplementation of ALA and LA on cell proliferation was also investigated.

Materials and Methods

Eagle MEM, RPMI1640 powder, and L-glutamine were purchased from Nissui Pharmaceutical Co., Ltd., (Tokyo, Japan). Streptomycin sulfate and potassium penicillin G were from Meiji Pharmaceutical Co., Ltd., (Tokyo, Japan). Fetal bovine serum (FBS) from Life Technologies, Inc. (Gaithersburg, U.S.A.), ALA (the purity of which was more than 98% and the peroxylipids content which was less than 0.1%) and dipalmitoylphosphatidylcholine (DPPC) from Sigma (St. Louis, U.S.A.), LA (the purity of which was more than 99% and the peroxylipids content which was less than 0.1%) from Nacalai Tesque (Kyoto, Japan), 2-hexyl-2-(10-methoxycarbonyldecyl)-4,4-dimethyl-3-oxazolidinyloxyl (12-MNS) from Aldrich (Milwaukee, U.S.A.), and [^{14}C]-ALA and [^{14}C]-LA from Amarsham Japan Co. (Tokyo, Japan), were all commercially available and used without further purification.

Cell Culture. Mouse fibroblast L-cells were maintained in Eagle MEM supplemented with 10% FBS and L-glutamine (292 $\mu g\,ml^{-1}$). Human colon cancer cells (RPMI4788 and BM314) were maintained in RPMI1640 supplemented with 10% FBS, streptomycin sulfate (100 $\mu g\,ml^{-1}$) and potassium penicillin G (200 units/ml).

Differential Scanning Calorimetry of Lipid Membrane. Calorimetric experiments were run on a Daini Seiko-sha SSC/560U by operating at a heating rate of 1.0 $°C\,min^{-1}$ with a range of 10 A. A sample for DSC measurements was prepared as follows: 50.0 μl of a chloroform solution containing 2.4 mg of DPPC (3.27×10^{-6} mol) with or without 0.4 mg of ALA (1.44×10^{-6} mol) or LA (1.43×10^{-6} mol) was added to a stainless-steel sample pan (SUS-70). After chloroform was removed under reduced pressure, 50.0 μl of pure water was added in the sample pan and the pan was sealed. The instrument was calibrated using benzoic acid as the standard. Each sample was repeatedly scanned three times in order to obtain complete reversibility. The phase-transition temperature was determined as T_c.

Electron Spin Resonance Measurements. A spin probe (12-MNS) was dissolved in methanol (2.5 $mg\,ml^{-1}$). A chloroform solution of DPPC (12.0 mg, 1.63×10^{-5} mol)

in the presence or absence of ALA (2.0 mg, 7.19×10^{-6} mol) or LA (2.0 mg, 7.18×10^{-6} mol) was mixed with 45.0 μl of a methanolic solution of 12-MNS (2.82×10^{-7} mol). After complete evaporation of the solvent under reduced pressure, the thin film thus obtained was swelled with 3.0 ml of a 20 mM Tris-HCl buffer (M=mol dm^{-3}) containing 200 mM NaCl (pH 8.6), and agitated on a Vortex mixer. The suspension thus-obtained was further sonicated under a nitrogen atmosphere using a probe-type sonifier (Tomy, UR-200) at 60.0 °C and 25 W for 15 min. ESR was run on a JEOL JES-FE1XG equipped with a temperature-control unit. The molecular motion of 12-MNS in a liposomal membrane is represented in terms of the rotational correlation time ($\tau_c^{(m^2)}$) when rapid and isotropic motion is observed (10^{-11} s$<\tau_c^{(m^2)}<10^{-9}$ s):[29,30]

$$\tau_c^{(m^2)} = 7.1\times10^{-10}W_0[R(-1)+R(+1)-2], \quad (1)$$

$$R(m) = W_m/W_0 = (h_0/h_m)^{1/2}, \quad (2)$$

where W_0 is the line width of the mid-field line in gauss, and h_{-1}, h_0, and h_{+1} are the respective peak heights of the triplet signal at the higher, mid, and lower magnetic fields.

The cells were coincubated with ALA or LA (0—80 μg ml^{-1}) by the same method as that employed for the assay of cytotoxicity (vide infra). The cells were incubated for 1 h (for 1×10^7 cells), 3 d (for 1×10^6 cells), and 5 d (for 5×10^5 cells). After a given incubation time, the adhered cells were stripped from the plate using 0.05% tripsin–0.02% EDTA in phosphate buffered saline (PBS) and centrifuged at $250\times G$ for 3 min. The obtained cell pellets were re-suspended in 2.0 ml of a Hanks balanced salt solution (HBSS(−)) and washed by centrifugation. Thereafter, the cell number was adjusted to 1×10^7 cells in 5.0 ml of HBSS(−). A cell suspension, which was preincubated at 37.0 °C for 10 min, was added to a thin film of a spin probe prepared by flashing gaseous nitrogen to a methanolic solution of the probe (1.02×10^{-7} mol). After coincubation at 37.0 °C for another 10 min, free 12-MNS (not internalized into the cells) was eliminated by centrifugation twice at $250\times G$ for 3 min, and the spin-labelled cells were supplied to ESR measurements.[31] Because the spin probe is partially immobilized in cell membranes and somewhat anisotropic, an approximate rotational correlation time (τ_0) is utilizable to determine the mobility change of the probe. The τ_0 is obtained from the following relationship:[32]

$$\tau_0 = 6.5\times10^{-10}W_0[(h_0/h_{-1})^{1/2}-1]. \quad (3)$$

Cell Uptake of Fatty Acid. The cell-uptake efficiency of fatty acids was evaluated using ^{14}C-labelled fatty acids (1.85—2.2 GBq mmol^{-1}). One ml of a cell suspension was placed on a 24-well microplate. The initial cell number was adjusted to 5×10^5 or 1×10^4 cells. Cells pre-cultured for 1 day at 37.0 °C were mixed with hot fatty acid (0—80 μg ml^{-1}) and incubated for another 1 h, 3 d, or 5 d. The culture medium was removed after a given incubation time, and cells were harvested by a treatment with 0.5 ml of 0.05% tripsin–0.02% EDTA in PBS. Free fatty acids were removed by means of velocity-gradient centrifugation using a mixed silicone oil (d=1.029).[33] A silicone oil mixture (0.50 ml, six parts of SH550 (d=1.07, Toray Silicone Co., Ltd.) and five parts of SH556 (d=0.980)) was placed in a 1.5 ml-volume Eppendorf tube and then formic acid (0.05 ml) was carefully injected at the bottom of the tube by using a microsyringe. A cell suspension (0.5 ml) was layered over the silicone oil layer. Free fatty acid remaining on the top layer was removed by centrifugation at $10{,}000\times G$ for 3 min. It was of course confirmed in advance that free fatty acid does not permeate through the silicone oil layer. After the tube was quickly frozen by immersing in dry ice–methanol (−78 °C), the frozen silicone oil layer was cut with a razor. The top layer, containing free fatty acid, and the bottom layer, containing the cells, were respectively replaced in 10 ml of a liquid scintillation cocktail (Clear sol I, Nacalai Tesque, Tokyo). The internalization efficiency of the fatty acid to the cells was calculated from the count ratio of the bottom layer to the total count of the radioactivity.

Assay of Cytotoxicity. A microscopic quantification assay was adopted for cytotoxicity. Cells on 1.0 ml of a medium containing 10% FBS were seeded in a 24-well microplate at 1×10^4 cells/well. The cells were then grown. A stock solution of ALA or LA was prepared by dilution of an ethanolic solution of the fatty acid (26.7 mg ml^{-1}) with physiological saline as for the final concentration to be 1.0 mg ml^{-1}. After incubation for 1 d at 37.0 °C under 5% CO_2–95% air, a given amount of ALA or LA solution was added into each well (0—80 μg ml^{-1}). The maximum ethanol content was kept below 0.3% (by vol) in each well. As a control experiment, cells were cultured in a medium supplemented with 0.3% ethanol. After incubation for 3 or 5 d, adhered cells were stripped from the microplate by treating with 0.05% tripsin–0.02% EDTA in PBS. The number of living cells in the detached cells was microscopically counted using the tripan blue exclusion method.

Results

Membrane Dynamics. The ESR spectrum of 12-MNS in DPPC and ALA-containing DPPC liposomal membranes at 25.0 °C are shown in Fig. 1. The ESR parameter, $-\log\tau_c^{(m^2)}$, obtained from the spectra, is plotted against the reciprocal of the incubation temperature ($1/T$) in Fig. 2. The addition of fatty acid into the liposomal membrane caused an increase in the $-\log\tau_c^{(m^2)}$ value; namely, an increase in the membrane fluidity. There was no significant difference in the effect on the membrane fluidity between ALA and LA added. Urano et al. investigated the effect of LA and ALA on the membrane fluidity of liposome as prepared from DPPC and dicetylphosphate by the fluorescence polarization method using 1,6-diphenyl-1,3,5-hexatriene as the probe,[34] and obtained exactly the same result as that observed in this work. An Arrhenius plot bent at the point which corresponds to the phase-transition temperature (T_c) of the lipid membranes. The T_c (39.4 °C) of the conventional DPPC liposome (Fig. 2) was almost identical with that obtained by other methods.[35,36] The addition of ALA into the liposome caused a decrease in T_c up to 32.1 °C, while that of LA decreased up to 31.2 °C (Fig. 2). Verma et al. also investigated the thermotropic behavior of a lecithin-fatty acid system by the Raman spectroscopic method.[37] The addition of ALA into DPPC liposome

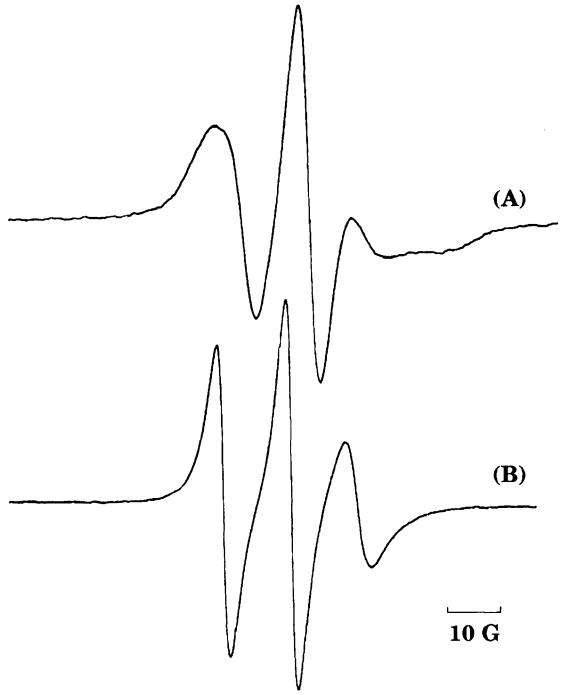

Fig. 1. ESR spectra of a spin probe, 12-MNS, in DPPC (A, top) and ALA-containing DPPC (B, bottom) liposomal membrane at 25.0 °C; lecithin : ALA : spin probe=57.8 : 25.5 : 1.0 by mol.

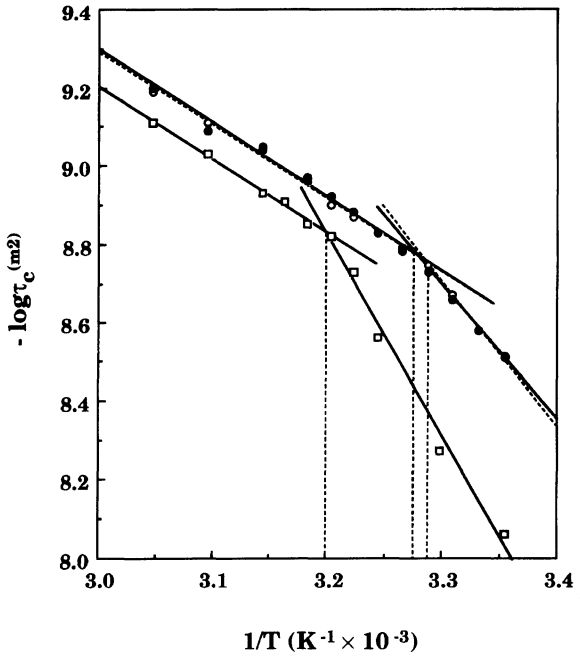

Fig. 2. Arrhenius plot of the negative logarithm of rotational correlation time ($-\log \tau_c^{(m^2)}$) for 12-MNS in DPPC liposomal membrane in the presence or absence of ALA or LA; lecithin : fatty acid : spin probe= 57.8 : 25.5 : 1.0 by mol. ○: LA-containing DPPC liposome, ●: ALA-containing DPPC liposome, and □: conventional DPPC liposome.

(DPPC : ALA = 1.0 : 0.6 by mol) shifted the transition temperature of the liposome from 41 to 32 °C, while LA shifted the temperature to 28 °C.[37] Table 1 shows the energy of activation of the rotational motion of the probe (ΔE) in liposomal membranes. No significant difference in ΔE among three liposomes at temperatures above T_c was observed. Below T_c, on the other hand, ΔE of LA or ALA-containing liposome was apparently smaller than that of the conventional liposome. However, no significant difference was observed between ALA and LA.

Phase Transition of the Membranes. The phase-transition temperatures obtained by DSC were 40.9 °C for DPPC liposome, 35.2 °C for ALA-embedded DPPC liposome, and 33.8 °C for LA-embedded one. A DSC analysis also demonstrated that the penetration of PUFAs into the liposomal membrane causes a decrease in the phase-transition temperature. Both measurements of ESR and DSC showed a good coincidence with each other.

Cell Uptake of PUFAs. The cell uptakes of ALA and LA are shown in Figs. 3 and 4. Figure 3 shows the amount of fatty acid internalized into the cells as a function of the dose within 1 h. No significant difference in the cell uptake was observed between ALA and LA. The uptake by BM314 was higher than that by RPMI4788. The uptake by mouse L-cell was less than that by the two tumor cells. In Fig. 4, the amounts of fatty acid taken up by cells for 3 and 5 d after the administration are shown for three cell lines. Both tumor cells took up more fatty acids than did the L-cell. Another interesting finding is that in all the cases LA was more internalized than ALA: 4.0-fold for L-cell and BM314 and 4.8-fold for RPMI4788 after 3 d incubation.

Fluidity of Plasma Membrane. In all previous ESR studies for L1210 murine leukemia,[5,6] B-16 melanoma,[28] macrophages,[38] mouse L-cell,[39] and human lymphocytes,[39] it has been confirmed that spin-labelled fatty acids always locate in the plasma membrane and are not internalized into cytosol. Kaplan et al. measured ESR spectra of L-cells labelled with 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl and 2-hexyl-2-(10-carboxydecyl)-4,4-dimethyl-3-oxazolidinyloxyl.[39] The ESR signal gradually decayed as a function of the incubation time. The ESR signal

Table 1. The Energy of Activation of the Rotational Motion (ΔE) of 12-MNS in DPPC Liposomal Membrane in the Presence or Absence of LA or ALA

	ΔE (kcal mol^{-1})	
	Below T_c	Above T_c
Conventional liposome	10.3	3.7
LA-Containing liposome	7.1	3.7
ALA-Containing liposome	6.7	3.8

1 cal=4.184 J.

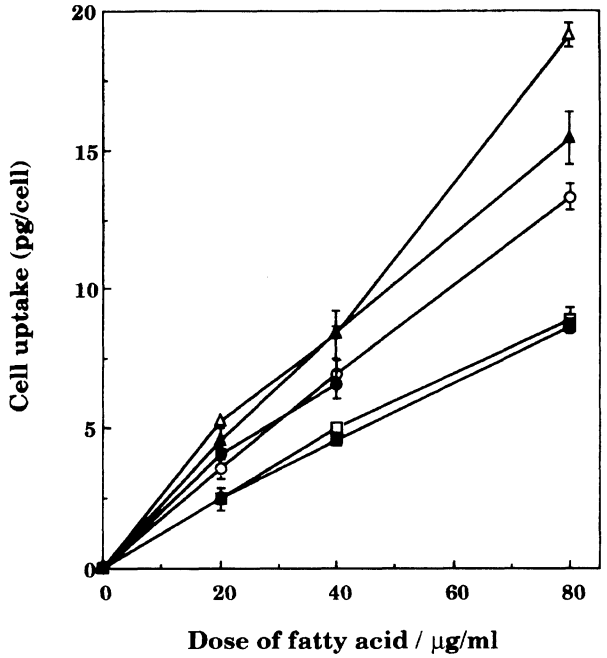

Fig. 3. Uptake of ALA (open symbol) and LA (closed symbol) by mouse fibroblast (L-cell) (□ and ■) and human colon cancer cells, RPMI4788 (○ and ●) and BM314 (△ and ▲) during the incubation for 1 h at 37.0 °C. Number of cells was 5×10^5 cells ml^{-1}.

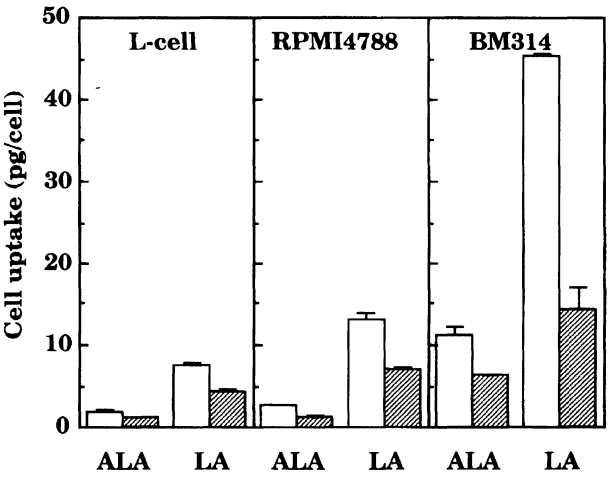

Fig. 4. Cell uptake of polyunsaturated fatty acids, ALA and LA, during the incubation at 37.0 °C for 3 d (□) and 5 d (▨). Initial cell number was 1×10^4 cells ml^{-1} and initial dose of fatty acid was 20 μg ml^{-1}.

almost completely restored when $K_3Fe(CN)_6$ was extracellularly added to the system after the signal had almost disappeared. It is, of course, known that the salt does not permeate into the cytosol. This means that the spin probe locates predominantly in the plasma membrane.[39] Simon et al. also measured the ESR spectra of L1210 murine leukemia cells and both the plasma membrane and the cytosol lipid droplet isolated from the cell using 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl as the spin probe. The ESR spectrum of the whole cell was almost the same as that of the plasma membrane, but was completely different from that of a lipid droplet. This result also suggests that the spin probe exists only in the plasma membrane.[5] Judging from these previous reports, it is reasonable to consider that the ESR signal of the cell as coincubated with 12-MNS certainly reflect the plasma membrane fluidity of the cell. The fluidity of the cell membranes may also possibly be changed by a change in the cell cycle accompanied by a perturbation with foreign materials. To more precisely understand the effect of the fatty acid on the cell-membrane fluidity, we therefore need to evaluate the difference in the τ_0 values between those cells treated with or without the fatty acid. This result shows that $\Delta\tau_0$ depends on both the sort of cell and the fatty acid employed (Table 2). When ALA or LA was added into a cell, $\Delta\tau_0$ increased; namely, the plasma membrane became more fluid upon the addition of the fatty acid, similarly to the case of an artificial liposomal membrane (Fig. 2). When 80 μg ml^{-1} of ALA was added to tumor cells, this effect was more drastic. However, the effect was not much in the case of a mouse L-cell. In addition, ALA made the tumor cell membrane more fluid than did LA, though this was not observed again in the mouse L-cell. Table 3 shows the change in the $\Delta\tau_0$ values as a function of the incubation time. Because ALA at doses higher than 40 μg ml^{-1} showed significant cytotoxicity and decreased the cell viability, the cells could not be recovered at all for incubations longer than 3 d (vide infra). Therefore, the amount of fatty acid added was kept constant at 20 μg ml^{-1} for investigating the effect of the fatty acid on the incubation period. The time-dependent fluidity change also indicated that ALA significantly increased the fluidity of the tumor cell membrane, especially of BM314. The extent of $\Delta\tau_0$ was maximum at 3 d.

Cytotoxicity of PUFAs. Figure 5 shows the cytotoxicity of ALA and LA to three cell lines. In coincubation for 5 d at a dose of 40 μg ml^{-1}, ALA drastically depressed the proliferation of the two tumor cells, especially that of RPMI4788. However, this was not the case for mouse fibroblast (L-cell). The administration of ALA at 80 μg ml^{-1} showed a strong cytotoxicity to all the cells examined, though the data are not shown. On the other hand, LA had no influence at all for cell proliferation, and rather accelerated the growth of BM314.

Discussion

In order to understand the effect of the two fatty acids on the cell-membrane fluidity, an ESR spin probe investigation was carried out using 12-MNS, because 12-MNS gives information about the relatively hydrophobic domain of the lipid bilayer membrane. The results revealed that ALA causes a significant increase in the membrane fluidity of colon cancer cells, but not much in the case of the mouse L-cell. Furthermore,

Table 2. Effect of Dose of ALA or LA on the Membrane Fluidity Change Represented by Rotational Correlation Time, $\Delta \tau_0$, of 12-MNS in Cell Membranes for 1 h at 37.0 °C

Fatty acid	Cell line	$\Delta \tau_0$/ns[a]		
		Concentration of fatty acid added/$\mu g\,ml^{-1}$		
		20	40	80
ALA	L-cell	0.12	0.21	0.37
	RPMI4788	0.26	0.27	1.08
	BM314	0.35	0.36	0.98
LA	L-cell	ND[b]	0.53	0.70
	RPMI4788	0.08	0.21	0.35
	BM314	0.13	0.24	0.76

a) $\Delta \tau_0 = \tau_0$ (in the absence of fatty acid) $- \tau_0'$ (in the presence of fatty acid).
b) ND: not determined.

Table 3. Effect of Incubation Time on the Membrane Fluidity Change at 37.0 °C ([Fatty Acid] = 20.0 $\mu g\,ml^{-1}$)

Fatty acid	Cell line	$\Delta \tau_0$/ns		
		Incubation time		
		1 h	3 d	5 d
ALA	L-cell	0.12	0.05	
	RPMI4788	0.26	0.31	−0.05
	BM314	0.35	0.62	0.34
LA	L-cell		−0.02	
	RPMI4788	0.08	0.12	−0.19
	BM314	0.13	0.22	−0.05

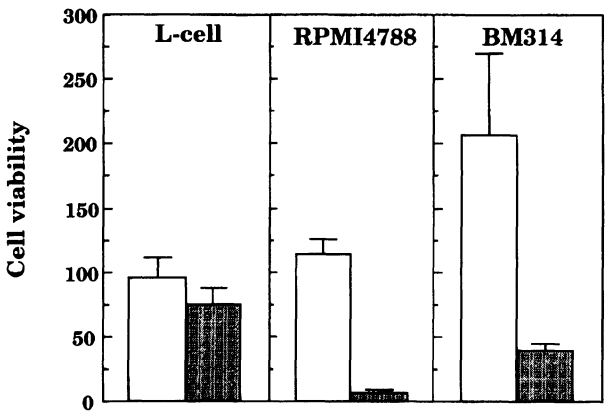

Fig. 5. Effect of LA (□) and ALA (■) on the viability of mouse fibroblast (L-cell) and human colon cancer cells (RPMI4788 and BM314) after incubation for 5 d at 37.0 °C; [fatty acid] = 40 $\mu g/10^4$ cells ml^{-1}.

the fluidity of the tumor cell membrane was more effectively affected by ALA than by LA. Other research groups have also reported that a lipid modification of the cell membrane results in a change in the membrane fluidity.[3—6]

Moreover, our findings suggest that the change in the cell-membrane fluidity cannot be simply explained in terms of the cell uptake efficiency of these unsaturated fatty acids. The uptake of LA by the cells was much higher than that of ALA in all of the cells examined. If the PUFAs content in the cell membrane directly correlates with the cell-membrane fluidity, LA must induce a larger fluidity change in the cell membrane than does ALA, because no significant difference was observed in the fluidity change of the DPPC model membrane between ALA and LA (Fig. 2).

The change in the membrane fluidity of intact cells does affect the activity of the membrane enzyme,[7] endocytosis,[8] and transport across the plasma membrane.[1,9] Therefore, incubation with PUFAs is also supposed to influence the change in the membrane fluidity, and, subsequently, the cell function, such as cell growth. Certainly, the membrane fluidity change of an intact cell induced by ALA was well correlated with the cytotoxic activity of ALA to the same cell. ALA showed a strong cytotoxicity to human colon tumor cells. Under the same condition, however, no significant toxicity was observed at all for a cloned mouse L-cell. LA rather promoted the growth of tumor cells under the same conditions. Bégin et al. have reported that both the ω-3 type fatty acids, except for docosahexaenoic acid, and the ω-6 type fatty acids selectively kill cancer cells, such as human prostatic (PC-3) adenocarcinoma, human lung (A-549), and breast (ZR-75-1) carcinomas at a dose of 20 $\mu g\,ml^{-1}$, but are safe for normal cells.[28] On the other hand, Rose and Connolly[40] have found that LA, ω-6 type fatty acids, stimulates the growth of several human breast cancer cell lines in a culture. This difference might result from a difference in the cell lines employed. Also, in several in vivo experiments, foods rich in the LA content enhanced the growth of cancer cells transplanted into animals,[13,14] while fish oil, which contains ω-3 type fatty acids, depresses tumor cell growth.[10—12] These previous in vivo experiments are basically coincident with our present results.

For more detailed discussion about the role of ALA, we must consider the influence of secondary metabolites brought about by a biochemical conversion of PUFAs.

PUFAs are precursors of both peroxylipids[15—17] and prostaglandins,[18—23] and their metabolites will also affect cell growth. Not only these points, but the intracellular location of these PUFAs may also be one of the important factors for elucidating the cytotoxicity.

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