Anticancer Activity of Free γ -Linolenic Acid on AH-109A Rat Hepatoma Cells and the Effect of Serum Albumin on Anticancer Activity of γ -Linolenic Acid *in Vitro*

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The cytotoxicity of γ -linolenic acid (C18:3 n -6) against rat hepatoma AH-109A cells and the effect of bovine serum albumin (BSA) on its toxicity were examined in culture. The proliferation of AH-109A cells, evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay, was significantly suppressed by γ -linolenic acid above 5 μ g/ml concentration in a serum-free culture medium. However, its toxicity was reduced by supplement of BSA. Similar observation of reduced toxicity by BSA was shown by the method of trypan blue dye exclusion and a colony formation assay. The cytotoxicity of γ -linolenic acid was correlated closely with the concentration of unbound (free) γ -linolenic acid. Production of thiobarbituric acid reactive material, one of the indicators of lipid peroxidation, was stimulated by γ -linolenic acid and inhibited by BSA. These results suggested that the presence of albumin suppressed the cytotoxicity of the free fatty acid.

Keywords — γ -linolenic acid; cytotoxicity; hepatoma cell; albumin; lipid peroxidation; *in vitro* study

Introduction

The anticancer effects of free fatty acids on the variety of malignant cells *in vitro*¹⁻⁶⁾ and *in vivo*⁶⁻⁸⁾ have been reported. In addition, selective toxicity of fatty acids toward cancer cells but not toward normal cells was suggested by recent studies.^{9,10)} Although several hypotheses have been proposed on mechanisms of anticancer effect of free fatty acids from various viewpoints,¹¹⁻¹⁴⁾ the exact mechanism is not yet clear. Moreover the study on the characteristics of free fatty acids as an anticancer drug does not seem to be sufficient.

In the present study, we examined the effect of serum albumin on the anticancer effect of γ -linolenic acid (C18:3 n -6), which has been shown to be the most potent and selective anticancer agent among the homologs. Serum albumin binds free fatty acids with very high affinity and transports them in body fluids¹⁵⁾ and its effect on anticancer activity of free fatty acids has not been studied sufficiently. We used a serum-free culture medium for the primary cell culture system of AH-109A rat hepatoma cells and examined (1) AH-109A cell growth cultured with

γ -linolenic acid, (2) viability of AH-109A cells and colony forming efficacy of AH-109A cells at several concentrations of γ -linolenic acid and exposure times, and (3) intracellular lipid peroxidation induced by γ -linolenic acid, associated with the presence of albumin.

Materials and Methods

Materials — Free γ -linolenic acid, approximately 98% pure, bovine serum albumin (BSA) fraction V without essential fatty acids were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Serum-free culture media, ASF-103 and ASF-104 were purchased from Ajinomoto Co., Tokyo, Japan. ASF-104 medium is albumin-free, while ASF-103 medium contains 1 mg/ml BSA.^{16,17)} 2-Thiobarbituric acid and 1,1,3,3-tetraethoxypropane were purchased from E. Merck Chemicals Co., West Germany, and Wako Pure Chemical Industries. Osaka, Japan, respectively. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Eastman Kodak Co., NY, U.S.A.

Cells — AH-109A rat hepatoma cells were

generously supplied from Dr. Ishimaru, Department of Pathology, Kumamoto University Hospital and were maintained by weekly i.p. inoculation of $1 \times 10^6/0.1$ ml AH-109A cells into 4-week-old female Donryu rats. For primary cell culture, AH-109A cells were obtained from tumor-bearing rat's ascites at 6–8 d after inoculation by discontinuous density gradient separation using Percoll.¹⁸⁾ AH-109A cells were washed twice with Hank's balanced salt solution and suspended in a test medium. Numbers of cells were determined by trypan blue exclusion. Cell culture was carried out at 37 °C in a 5% CO₂-humidified incubator in all experiments.

Growth Suppressive Effect of γ -Linolenic Acid — AH-109A cells were suspended at 1.0×10^6 cells per ml in ASF-104 medium in the presence of albumin at several concentrations or in ASF-103 medium. An ethanolic solution of γ -linolenic acid was added to the AH-109A cell suspension so that the final ethanol concentration became 0.2%. Then the cells were seeded at 1.0×10^4 cells per well in a 96-well microplate and cultured for 24 h. Cell growth was determined by a MTT assay. The MTT assay was carried out according to the Mosmann's method¹⁹⁾ for the cells before γ -linolenic acid was added (day 0) and the cells cultured for 24 h (day 1). Briefly, 25 μ l of 5 mg/ml MTT solution in phosphate-buffered saline (PBS), pH 7.0, was added to each well and cells were cultured for 4 h. Then, the supernatant was discarded and isopropyl alcohol-HCl (0.04 N, 150 μ l) was added to dissolve blue crystals of formazan, generated from MTT, and absorbance of each well was measured by a microplate reader using a test wavelength of 550 nm and a reference wavelength of 630 nm. Linearity between number of cells and absorbance, extended from 5×10^3 to 1×10^5 cells/well. The growth rate was calculated as the percent of absorbance at day 1 to the absorbance at day 0.

Cell Viability at Several γ -Linolenic Acid Concentrations and Exposure Times — AH-109A cells were suspended at 1×10^6 cells per ml in ASF-103 medium and seeded in a 25-cm² flask. Then an ethanolic solution of γ -linolenic acid was added so that the final ethanol concentration became 0.2% and the cells were

incubated. Aliquots of the cell suspension were withdrawn at several exposure times and the number of viable cells and nonviable cells were measured by trypan blue exclusion. A survival fraction was calculated as the ratio of viable cells to total cells.

Colony Forming Efficacy after Exposure to γ -Linolenic Acid — Exposure of AH-109A cells to γ -linolenic acid was carried out similarly to "Cell Viability at Several γ -Linolenic Acid Concentrations and Exposure Times". Aliquots of cell suspension were withdrawn and colony forming efficacy of the cells was determined by the double layer soft agar culture system according to the method of Hinuma *et al.*^{20,21)} Briefly, AH-109A cells were suspended at 5×10^2 cells per 0.5 ml in an upper layer medium (ASF-103 medium containing 10% fetal bovine serum and 0.33% agar) and the suspension was introduced on a lower layer medium (0.5 ml, ASF-103 medium containing 10% fetal bovine serum and 0.4% agar) and cultured (day 0). Colonies were counted on the 14th day. The number of colonies was about 150/well in a control group.

Lipid Peroxidation — Intracellular thiobarbituric acid-reactive material (TBARM) content after γ -linolenic acid exposure for 8 h with or without albumin was measured for estimation of lipid peroxidation according to the Begin's method.¹¹⁾ Briefly, separated cells were washed twice with saline and suspended in saline at 5×10^5 cells per ml. Trichloroacetic acid solution (20%, 2 ml) and thiobarbituric acid (0.67%, 2 ml) were added to the cell suspension and incubated for 20 min at 100 °C. Then the mixture was centrifuged at 12000 *g* for 10 min at 4 °C. The fluorescent intensity of the supernatant was measured with excitation at 534 nm and emission at 564 nm. TBARM was expressed as picomoles of malondialdehyde-equivalent/ 10^4 cells.

γ -Linolenic Acid Analysis — The culture medium was passed through an ultrafilterable membrane. γ -Linolenic acid in the filtrate was extracted with chloroform-methanol (2:1) containing 0.02% butylated hydroxytoluene and heptadecanoic acid (C17:0, internal standard). The organic layer was collected and evaporated under reduced pressure. The residue was dissolved in methanol containing trimethylanilini-

TABLE I. Growth Rate of AH-109A in Serum-Free Culture Medium in the Presence of γ -Linolenic Acid

Concentration of γ -linolenic acid ($\mu\text{g/ml}$)	Culture medium				
	ASF-104	ASF-104 + BSA 0.1 mg/ml	ASF-104 + BSA 0.5 mg/ml	ASF-104 + BSA 1 mg/ml	ASF-103
0	174.1 $\pm$ 4.2	148.7 $\pm$ 5.1	166.3 $\pm$ 3.3	174.7 $\pm$ 3.9	213.2 $\pm$ 1.2
1	204.9 $\pm$ 7.9	263.6 $\pm$ 4.0	253.8 $\pm$ 10.3	274.0 $\pm$ 7.8	216.2 $\pm$ 1.2
5	97.2 $\pm$ 21.0	182.5 $\pm$ 32.0	238.6 $\pm$ 7.1	270.8 $\pm$ 3.9	205.8 $\pm$ 3.5
10	39.2 $\pm$ 1.4	26.0 $\pm$ 5.7	159.8 $\pm$ 17.9	222.1 $\pm$ 2.6	197.7 $\pm$ 5.2
20	31.4 $\pm$ 1.4	22.7 $\pm$ 5.6	13.0 $\pm$ 0.5	98.8 $\pm$ 2.6	179.8 $\pm$ 5.8

Values were percent absorbance at day 1 to the absorbance at day 0 and mean $\pm$ S.E. of three experiments.

um hydroxide (methylation agent).²²⁾ The free γ -linolenic acid concentration in the culture medium was measured by gas chromatography-mass spectrometry (GC-MS, DX-303 DA-5000 system, JEOL, Tokyo, Japan). Aliquots of the solution were introduced into GC-MS. GC-MS condition was as follows: The coiled glass column (1.0 m $\times$ 3 mm i.d.) of the gas chromatograph was packed with 5% diethyleneglycol succinate- H_3PO_4 on Chromosorb W (60–80 mesh). The injector, separator, and inlet temperature were 300, 270, and 280 $^\circ\text{C}$, respectively. The column temperature was raised from 180 to 210 $^\circ\text{C}$ for 3 min. The carrier gas was helium and the flow rate was 30 ml/s. The ionization mode was electron impact method. Selected ion monitoring, m/z 292.0 for methyl γ -linolenate and m/z 284.0 for methyl heptadecanoate (internal standard), was performed. The total free

γ -linolenic acid concentration in the culture medium was similarly measured without ultrafiltering. γ -Linolenic acid was stable in the all culture media used under cell-free condition for 48 h.

Results

Effect of γ -Linolenic Acid on the Growth Rate of AH-109A Cultured Cells in ASF-103 and ASF-104 Media

Table I shows growth rates of AH-109A cells cultured in the presence of γ -linolenic acid. In the case where a culture medium was ASF-104 (BSA free), growth suppressive effect was observed with more than 5 $\mu\text{g/ml}$ of γ -linolenic acid. The addition of BSA into ASF-104 medium made the suppressive effect of γ -linolenic acid less effective. The suppressive effect was ob-

Fig. 1. The Effect of γ -Linolenic Acid on AH-109A Cell Viability in ASF-103 Medium

(A) Relationship between total γ -linolenic acid concentrations and surviving fraction. (B) Relationship between albumin-unbound γ -linolenic acid concentrations and surviving fraction. Exposure time: ○, 6 h; △, 12 h; □, 24 h. All points are mean $\pm$ S.E. of 6 experiments.

Fig. 2. The Effect of γ -Linolenic Acid on the Colony Forming Efficacy of AH-109A Cells in ASF-104 (BSA Free) Medium

The colony forming efficacy was expressed as the percent of number of colonies to control. Exposure time: ○, 1 h; ▼, 2 h; □, 4 h; ▽, 7 h. All points are mean $\pm$ S.E. of 6 experiments.

served with more than 10 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ of γ -linolenic acid in ASF-104 medium containing BSA at 0.1 and 0.5 mg/ml, respectively. In ASF-104 medium containing BSA at 1.0 mg/ml, growth suppressive effect was hardly observed with less than 20 $\mu\text{g/ml}$ of γ -linolenic acid. In ASF-103 medium (containing BSA at 1 mg/ml), mild suppressive effect was observed with more than 10 $\mu\text{g/ml}$ of γ -linolenic acid.

Cell Viability at Several γ -Linolenic Acid Concentrations and Exposure Times in ASF-103 Medium

Figure 1A shows survival fractions of AH-109A cells at several γ -linolenic acid concen-

trations and exposure times in ASF-103 medium. Cytotoxicity of γ -linolenic acid was not observed with less than 20 $\mu\text{g/ml}$ and dramatically observed with more than 20 $\mu\text{g/ml}$ at three exposure times. Figure 1B shows the relationship between surviving fractions and the initial concentrations of albumin-unbound γ -linolenic acid in the medium. Cytotoxicity was related to the concentration of albumin-unbound γ -linolenic acid. The concentration of albumin-unbound γ -linolenic acid was low at less than 20 $\mu\text{g/ml}$ of total γ -linolenic acid and cytotoxicity of γ -linolenic acid was weak under these conditions. The concentration of albumin-unbound γ -linolenic acid was increased as the concentration of total γ -linolenic acid was increased to more than 20 $\mu\text{g/ml}$ and the survival fraction was decreased.

Colony Forming Efficacy of AH-109A Cells at Several γ -Linolenic Acid Concentrations and Exposure Times in ASF-103 and ASF-104 Media

Figure 2 shows the colony forming efficacy of AH-109A cells at several γ -linolenic acid concentrations and exposure times in ASF-104 (BSA free) medium. The logarithmic plot of percent of number of colonies to control *versus* γ -linolenic acid concentration exhibited linearity and cytotoxicity of γ -linolenic acid was increased as the exposure time was prolonged.

Figure 3A shows the colony forming efficacy of AH-109A cells in ASF-103 medium at several γ -linolenic acid concentrations and exposure times. Cytotoxicity was observed above the γ -linolenic acid concentration of 10–20 $\mu\text{g/ml}$ at

Fig. 3. The Effect of γ -Linolenic Acid on the Colony Forming Efficacy of AH-109A Cells in ASF-103 Medium

The colony forming efficacy was expressed as the percent of number of colonies to control. (A) Relationship between total γ -linolenic acid concentration and colony forming efficacy. (B) Relationship between albumin-unbound γ -linolenic acid concentration and colony forming efficacy. Exposure time: ○, 2 h; △, 7 h. All points are mean $\pm$ S.E. of 6 experiments.

Fig. 4. Intracellular TBARM of AH-109A Cells Induced by γ -Linolenic Acid in ASF-104 Medium in the Presence of BSA at 0.25 mg/ml

Concentration of γ -linolenic acid: □, 1 μ g/ml; Δ, 5 μ g/ml; ○, 10 μ g/ml. All values were mean $\pm$ S.E. of three experiments.

each exposure time. Figure 3B shows the relationship between colony forming efficacy and albumin-unbound γ -linolenic acid concentration in the medium before the culture was started. Cytotoxicity was related to an albumin-unbound γ -linolenic acid concentration. These data were similar to observed phenomena in Fig. 1A and B.

Lipid Peroxidation Induced by γ -Linolenic Acid

Figure 4 shows the intracellular TBARM of AH-109A cells induced by the exposure to several γ -linolenic acid concentrations in ASF-104 medium in the presence of BSA at 0.25 mg/ml. TBARM hardly increased within 8 h below the γ -linolenic acid concentration of 5 μ g/ml. At the γ -linolenic acid concentration of 10 μ g/ml, however, TBARM increased after 2 h. The colony forming efficacy of AH-109A cells, the values of intracellular TBARM and the consumption of γ -linolenic acid at 8 h in Fig. 4 are summarized in Table II. The consumption of γ -linolenic acid

Fig. 5. The Effect of BSA on Intracellular TBARM of AH-109A Cells Induced by γ -Linolenic Acid at 5 μ g/ml

Concentration of BSA: ○, 100 μ g/ml; Δ, 250 μ g/ml; □, 500 μ g/ml. All values were mean $\pm$ S.E. of three experiments.

was calculated from the amount of γ -linolenic acid remaining in the medium at 8 h. There were no significant differences in consumption of γ -linolenic acid, TBARM and colony forming efficacy between two groups supplemented with γ -linolenic acid at 1 and 5 μ g/ml. In the group supplemented with γ -linolenic acid at 10 μ g/ml, however, the consumption of γ -linolenic acid and intracellular TBARM increased and colony forming efficacy decreased.

Figure 5 shows the intracellular TBARM of AH-109A cells induced by the exposure to 5 μ g/ml γ -linolenic acid in ASF-104 medium containing BSA at several concentrations. The values of TBARM at each time point were decreased as the BSA concentration was increased. The colony forming efficacy of AH-109A cells as the percent to control at 8 h was 63.8 ± 3.2 , 93.8 ± 2.1 , and 100.0 ± 2.8 , for the BSA concentration of 0.1, 0.25, and 0.5 mg/ml, respectively.

TABLE II. Consumption of γ -Linolenic Acid, Intracellular TBARM and Colony Forming Efficacy at 8 h in Fig. 4

Concentration of γ -linolenic acid (μ g/ml)	Consumption of γ -linolenic acid (μ g/ 10^4 cells)	Intracellular TBARM (pmol/ 10^4 cells)	Colony forming efficacy (%)
0	—	<1.0	100
1.0	0.105 ± 0.013	1.33 ± 0.14	105.6 ± 5.5
5.0	0.094 ± 0.039	3.44 ± 0.69	100.0 ± 4.8
10.0	$0.295 \pm 0.027^a)$	$12.29 \pm 2.13^a)$	$53.1 \pm 2.0^b)$

Values were mean $\pm$ S.E., $n = 3$. a) $p < 0.05$. b) $p < 0.01$.

Discussion

Free fatty acids in body fluids are bound to albumin with high affinity and transported in systemic circulation.¹⁵⁾ In general, drugs bound to albumin are expected to be pharmacologically less potent. Bégin *et al.*¹¹⁾ suggested that the ability of free unsaturated fatty acids to kill tumor cells was correlated with their ability to stimulate generation of products of lipid peroxidation. Badwey *et al.*²³⁾ reported earlier that release of superoxide anion from the human neutrophils by free unsaturated fatty acid was prevented by addition of albumin. Nilausen²⁴⁾ reported that serum albumin combined with fatty acids had considerable growth promoting effect on cultured hamster cells. It is suggested from these viewpoints that serum albumin suppressed the anticancer effect of free unsaturated fatty acids. Therefore we examined the effect of serum albumin on the anticancer effect of γ -linolenic acid using a serum-free cell culture system.

In the present study, serum albumin reduced the anticancer effect of γ -linolenic acid on AH-109A cell growth; the growth rate of AH-109A was rather increased by supplement with BSA and γ -linolenic acid (Table I). Only BSA combined with fatty acid increased the growth rate of AH-109A while the addition of essential fatty acid-free BSA did not increase the growth rate. These results were in agreement with Nilausen's report. Serum albumin also reduced anticancer effect of γ -linolenic acid on AH-109A cell viability and on the colony forming efficacy of AH-109A cells. Moreover, concentrations of albumin-unbound γ -linolenic acid were closely related to anticancer effect of γ -linolenic acid. Intracellular TBARM, which is expected to be an indicator of lipid peroxidation induced by γ -linolenic acid was decreased as the ratio of albumin concentration to γ -linolenic acid concentration was increased in the two systems in which either the albumin concentration or the γ -linolenic acid concentration was fixed. The colony forming efficacy of AH-109A cells was decreased and the consumption of γ -linolenic acid was increased as the intracellular TBARM was increased. From these results, it is suggest-

ed that the anticancer effect of γ -linolenic acid was suppressed by binding to serum albumin and albumin-unbound γ -linolenic acid was a potent cytotoxic agent to cancer cells.

On the other hand, there have been many reports which demonstrated the anticancer effect of fatty acids *in vivo*. The fatty acids in these studies were not only free fatty acids but also esterified fatty acids with ethanol,^{3,9,10)} methanol,⁸⁾ and glycerol.²⁵⁻²⁷⁾ One of the mechanisms suggested in these studies is the modification of fatty acid components in cell membranes.^{11,12,28)} In such a system, the effect of serum albumin on the anticancer effect of fatty acids might be different from the results of the present study, and it is a problem to be examined in a further study.

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