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Abstract

It was investigated to clarify the relationship between the composition of the lipid fractions obtained from the ascitic fluid of Ehrlich ascites tumor bearing mice and its uncoupling activity after whole body irradiation (1,000 r). 1. Oxidative phosphorylation of Ehrllch ascites tumor cells was loosely uncoupled with the addition of ascitic lipid fraction extracted from tumor bearing mice. 2. The uncoupling activity of the lipid fraction on the oxidative phosphorylation of the tumor cells increased after whole body irradiation. 3. Ascitic lipid fraction, especially acetone soluble fraction accelerated mitochondrial swelling, and the swelling action was increased remarkably by the whole body irradiation. 4. No significant changes were observed in the proportion of acetone soluble fraction to acetone insoluble fraction in the ascitic lipid after X-irradiation, and the proportion of the both fractions was approximately 9 : 1, respectively. 5, Main compositions of total and non-esterified fatty acids in the ascitic fluid obtained from the control and X-irradiated groups were palmitic, stearic, oleic, linoleic and palmitoleic acids, and the proportions of unsaturated acids, especially oleic and linoleic acids in both fatty acid fractions were greater in the X-irradiated group. 6. Remarkable increment of unsaturated fatty acid especially linoleic acid, was also observed in the total fatty acids of the tumor cells separated from the X-irradiated group. 7. It can be concluded that an uncoupling agent extracted from ascitic fluid of the X-irradiated group was a mixture of long-chain fatty acids, especially oleic and linoleic acids. 8. It was also discussed that uncoupling oxidative phosphorylation in liver mitochondria after whole body irradiation may be caused by a similar mechanism to that in the turnor cells.

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UNCOUPLING AGENT OF OXIDATIVE PHOSPHORYLATION FROM ASCITIC FLUID OF TUMOR BEARING MICE AFTER X-IRRADIATION*

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It was reported previously[1] that the oxidative phosphorylation of Ehrlich ascites tumor cells was inhibited and the degree of the inhibition increased in proportion to the time for 24 hours after whole body irradiation (1,000 r). But the inhibitory action was not observed in the case of direct irradiation to the isolated tumor cell suspension (*in vitro*). However, the oxidative phosphorylation of the isolated tumor cells was uncoupled by the addition of the ascitic fluid obtained from the whole body irradiated tumor bearing mice. This uncoupling activity was found in the fraction of heat stable, ethanol soluble lipid of the ascitic fluid. The uncoupling activity of this fraction was much stronger in that from the X-irradiated group than in the control. These findings suggest that the uncoupling action of X-ray is indirect and induced as a result of the changes in the fatty acid composition of ethanol soluble lipids in the ascitic fluid.

In the present paper, the relationship between the fatty acid composition of the lipid fraction obtained from the ascitic fluid and its uncoupling activity, and the mechanism of uncoupling action of X-ray have been described.

MATERIALS AND METHODS

Materials : Ascitic fluid, tumor cells and livers were separated from Ehrlich ascites tumor bearing mice 6 hours after whole body irradiation of 1,000 r with the same procedures as those described in previous paper[1].

Extraction and fractionation of total lipids : Total lipids were extracted from ascitic fluid, tumor cells and livers by the method of FOLCH *et al*[2].

Total lipids of the ascitic fluid were separated into acetone soluble and insoluble fractions with addition of 20 volumes of acetone to a volume of the lipid

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fraction. Both fractions were used as the acetone soluble- and insoluble-lipids in the experiment.

Uncoupling activity of lipid fraction : Uncoupling activity of the ascitic total lipid fraction on Ehrlich ascites tumor cells was measured by Warburg apparatus and incubation mixture was as follows; 1.7 ml of tumor cell suspension, 0.2 ml of 20 per cent KOH in the center well, 0.6 ml of Krebs-Ringer phosphate solution contained 30 μc of P^{32} in side arm (a) and 0.5 ml of ascitic total lipid fraction (eq. 1.25 ml of ascitic fluid) emulsified with 0.1 per cent Tween 80-saline solution in side arm (b).

The incubation was carried out at 38°C for 30 minutes with air environment.

Mitochondrial swelling by lipid fractions : Mitochondria were isolated from rat liver by the method of HOGEBOOM[3], and were washed 3 times with 0.25 M sucrose. One g tissue equivalent of these mitochondria was suspended in 1 ml of 0.25 M sucrose as stock mitochondria. Freshly prepared rat liver

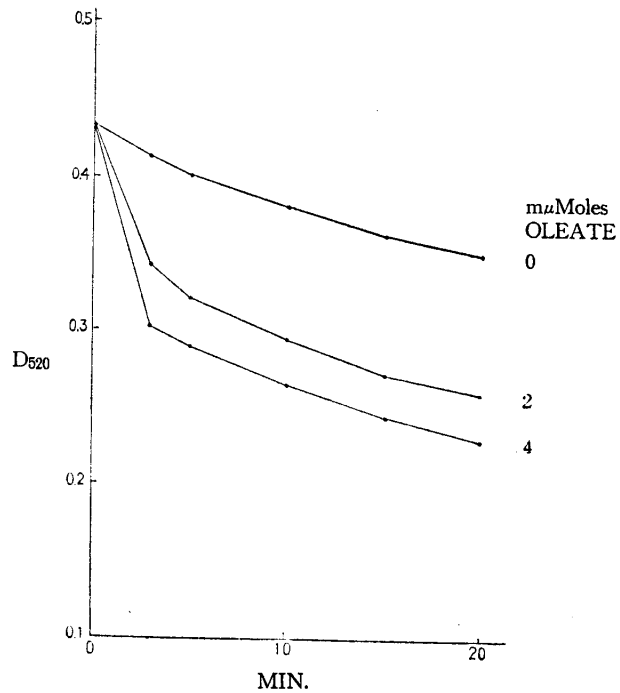

Fig. 1 Effect of various concentrations of sodium oleate on swelling of mitochondria. Freshly prepared rat liver mitochondria were added to medium containing 5 ml of 0.125 M KCl-0.02 M Tris-HCl buffer (pH 7.4) to give an initial absorbancy of about 0.5 at 520 mμ. Sodium oleate were dissolved in 0.2 ml of ethanol and added at zero time. Swelling was measured at 20° for 20 minutes.

mitochondria were added to 5 ml of 0.125 M KCl-0.02 M Tris-HCl buffer solution (pH 7.4) to give an initial absorbancy of about 0.5 at 520 mμ. An aliquot of ascitic lipid was dissolved in 0.2 ml of ethanol and added to the medium at zero time. The incubation was carried out at 20°C, and the extinction of the incubation mixture was measured with Beckman spectrophotometer at 520 mμ for the period of 20 minutes from the start. The mitochondria used in the experiment manifested the swelling by the addition of sodium oleate as shown in Fig. 1.

Chromatography of fatty acids : The chloroform-methanol fractions extracted from the ascitic fluid, tumor cells and livers were taken to dryness under a stream of nitrogen gas with reduced pressure and were saponified for 2 hours with 20 ml of 1 N NaOH-ethanol solution under a stream of nitrogen gas. The solution was extracted three times with 30 ml of ether. The final extract was evaporated to dryness. This fraction was used as total fatty acids of the ascitic fluid.

Non-esterified fatty acids were separated as follows: The acetone soluble lipid fraction of the ascitic fluid, dissolved in 4 ml of 96 per cent ethanol, was added 1 ml of 5 N KOH and 25 ml of water. The solution was extracted three times with 30 ml of ether, and the alkaline aqueous layer was acidified with HCl to pH 1—2 and then extracted three times with ether. The final extract was evaporated to dryness. This fraction was used as non-esterified fatty acids of the ascitic fluid.

Both fractions of total and non-esterified fatty acids were esterified with diazomethane. These methylesters of fatty acids were separated and identified by gas-liquid chromatography. A Shimazu model GC-1B gas chromatograph with a hydrogen flame ionization detector was used. Columns (2.25 m) were packed with 10 per cent succinate polyester on shimalite, 60 to 80 mesh, and the columns were operated at 220° with nitrogen gas. Flow rate of the carrier gas was 50 to 60 ml per minute. Identification of peaks of the chromatogram was aided by co-chromatography of standard fatty acid methyl esters. When standards were not available, peaks were tentatively identified by relative retention time.

RESULTS

Uncoupling of oxidative phosphorylation by ascitic lipid fraction : In order to test the properties of ascitic lipid as an uncoupler, the action of ascitic total lipid fraction was observed by measuring O_2 uptake and incorporation of radioactive phosphate into *A* 10 phosphate fraction of tumor cells. As shown in Table 1, O_2 uptake and incorporation of radioactive phosphate into *A* 10 phosphate

fraction decreased to 68 and 51 per cent, respectively, by the addition of ascitic total lipid extracted from the X-irradiated group. Then, relative P/O ratio

Table 1 Effect of lipid fractions on the oxygen uptake and incorporation of P^{32} into Δ 10 P fraction of Ehrlich ascites tumor cells under endogenous substrate.

Incubation mixture was as follows; 1.7 ml of tumor cell suspension (20,000,000 cells/ml of Krebs-Ringer solution), 0.6 ml of Krebs-Ringer phosphate buffer (30 μc of P^{32}, pH 7.4), 0.5 ml of total ascitic lipid (eq. 3.75 ml of ascitic fluid). The incubation was carried out at 38° for 30 minutes. Irradiated lipid fraction was extracted from the ascitic fluid of the tumor bearing mice, which were transplanted Ehrlich ascites tumor cells in the peritoneal cavities of Swiss albino mice (30 animals, ♂), 6 hours after whole body irradiation (1,000 r).

Lipid fraction added	Oxygen uptake	Radioactivity of Δ 10 P fraction	Relative P/O ratio
	μl/hr. /flask	cpm/hr. /flask	%
None	205	13347	100
Control lipid fraction	151	8537	88
Irradiated lipid fraction	140	6595	71

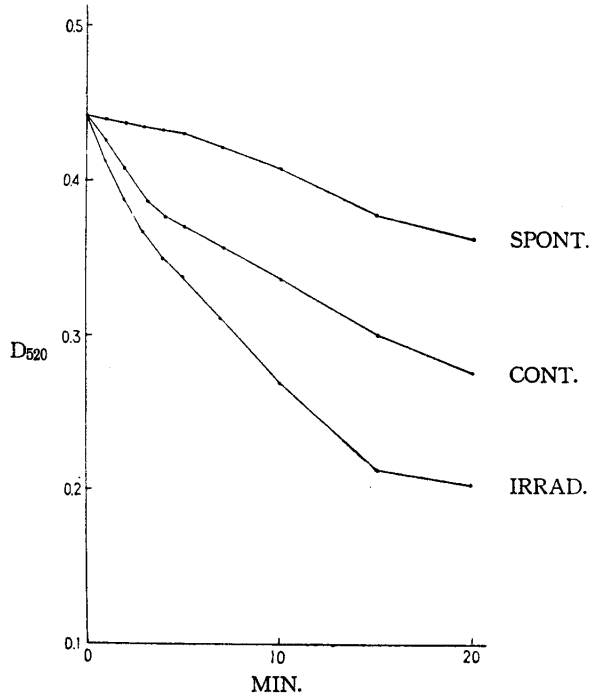

Fig. 2 Swelling action of total lipid fraction of ascitic fluid on rat liver mitochondria. Total lipid fractions (eq. 0.62 ml of ascitic fluid) were dissolved in 0.2 ml of ethanol and added to medium at zero time. The details were described in the text and Fig. 1. SPONT., spontaneous swelling; CONT., ascitic fluid from control group; IRRAD., ascitic lipid from X-irradiated group.

decreased to 71 per cent.

Effect of ascitic lipid fraction on the mitochondrial swelling : It is well known that the uncoupler of oxidative phosphorylation like oleic acids displays the swelling action on rat liver mitochondria (Fig. 1).

Fig. 2 shows that total lipid fraction extracted from the ascitic fluid causes acceleration of swelling of rat liver mitochondria, and the swelling action was more remarkable in the fraction extracted from X-irradiated group.

In order to clarify the properties of an uncoupler in the total lipid fraction, the ascitic lipid was separated into acetone soluble and insoluble fractions.

Table 2 shows the proportions of the acetone soluble and insoluble fractions to the total lipids of the control and X-irradiated groups. There was no significant difference in the proportion between them, namely, the proportion of the acetone soluble lipid to the acetone insoluble lipid was about 9 : 1, respectively. The swelling action of the acetone soluble lipid on rat liver mitochondria was more remarkable in that extracted from the X-irradiated group as shown in Fig.

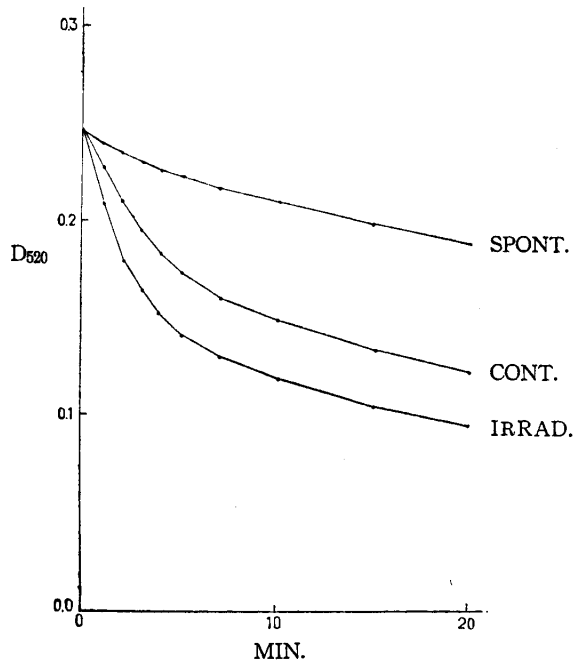

Fig. 3 Swelling action of acetone soluble lipid fractions of ascitic fluid on rat liver mitochondria. Acetone soluble lipid (430 μg.) was dissolved in 0.2 ml of ethanol and added to medium at zero time. The details were described in the text and Fig. 1. SPONT., spontaneous swelling; CONT., acetone soluble lipid of ascitic fluid from control group; IRRAD., acetone soluble lipid of ascitic fluid from X-irradiated group.

Table 2 Effect of X-irradiation on the composition of total fatty acids of ascitic fluid in the tumor bearing mice.

Control: total fatty acids of control group. Irradiated: total fatty acids of ascitic fluid obtained from the mice 6 hours after whole body irradiation. The details were described in the text and Table 1.

Group	Acetone soluble lipid fraction	Acetone insoluble lipid fraction
	%	%
Control	89.5	10.5
Irradiated	90.6	9.4

3, but there was no difference in that of the acetone insoluble lipids between them.

Changes in fatty acid composition with X-irradiation : Gas chromatography of the lipid fractions was carried out to clarify the difference in the fatty acid compositions between the X-irradiated and non-irradiated groups.

Table 3 shows that the main components of the total fatty acids in the

Table 3 Effect of X-irradiation on the composition of total fatty acids of ascitic fluid in the tumor bearing mice

Group*	Percentage composition							Ratio of unsaturated F.A./saturated F.A.
	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:2'}$	$C_{20:poly.}$	
Control	64.2	11.8	13.9	6.8	3.2	—	trace	0.28
Irradiated	41.7	13.3	15.8	17.6	9.5	1.9	trace	0.74

* Control: the ascitic fluid obtained from the tumor bearing mice starved for 6 hours. Irradiated: the ascitic fluid obtained from the tumor bearing mice starved for 6 hours after whole body irradiation.

ascitic fluid were palmitic, palmitoleic, stearic, oleic and linoleic acids. The proportion of unsaturated fatty acids, especially oleic and linoleic acids, to saturated fatty acids of the ascitic fluid increased with the whole body X-irradiation. Similar pattern was found in the case of the non-esterified fatty acids in the ascitic fluid as shown in Figs. 4 (a) and (b).

As it has been clarified that the proportion of unsaturated fatty acids in the ascitic lipids was remarkably increased with whole body X-irradiation, gas chromatography of fatty acids in the tumor cells was carried out to clarify whether the composition of fatty acids in the tumor cells are affected with these changes in the ascitic fluid. Table 4 shows that the level of linoleic acid increased in contrast with the decrease in level of stearic acid of the X-irradiated group.

Gas chromatography of fatty acids of the liver was carried out to clarify whether the increment of unsaturated fatty acid level in the ascitic fluid correlates

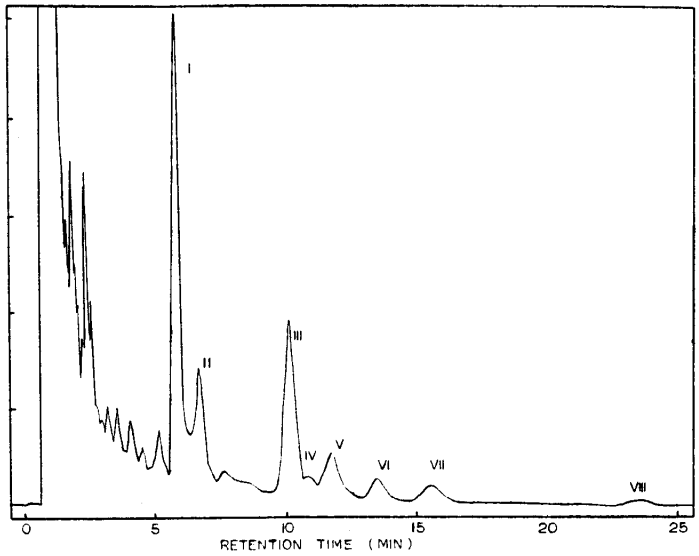

Fig. 4 (a) Typical gas-liquid chromatogram of the non-esterified fatty acids in the ascitic fluid of the control mice. The ascitic fluid was obtained from the tumor bearing mice starved for 6 hours. Peak I, palmitic acid; Peak II, palmitoleic acid; Peak III, stearic acid; Peak IV, oleic acid; Peak V, linoleic acid; Peak VI, octadeca-dienoic acid; Peak VII, linolenic acid; Peak VIII, eicosa-polyenoic acid.

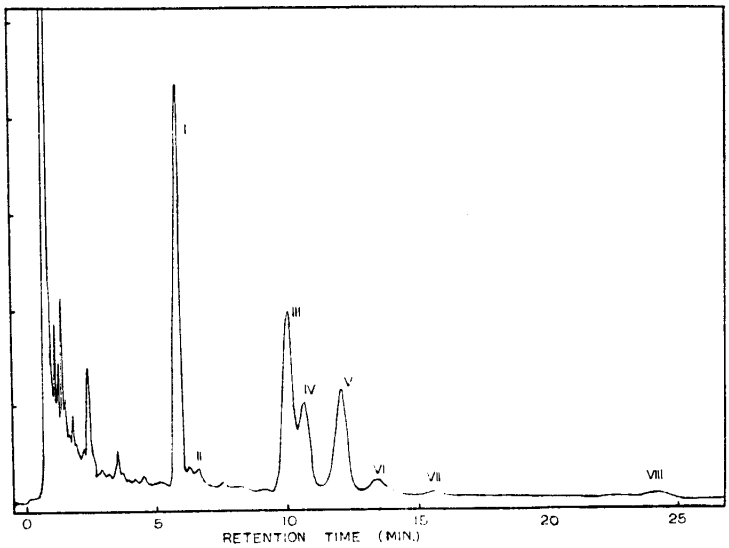

Fig. 4 (b) Typical gas-liquid chromatogram of the non-esterified fatty acids in the ascitic fluid of the X-irradiated mice. The ascitic fluid was obtained from the tumor bearing mice starved for 6 hours after whole body irradiation. Peak I, palmitic acid; Peak II, palmitoleic acid; Peak III, stearic acid; Peak IV, oleic acid; Peak V, linoleic acid; Peak VI, octadeca-dienoic acid; Peak VII, linolenic acid; Peak VIII, eicosa-polyenoic acid.

Table 4 Effect of X-irradiation on the composition of total fatty acids of Ehrlich ascites tumor cells in the tumor bearing mice

Group*	Percentage composition						
	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:20}$	$C_{20:poly.}$
Initial	26.3	4.9	20.5	22.4	22.2	—	1.6
Control	24.3	7.3	18.6	19.8	21.1	3.9	1.9
Irradiated	25.2	3.6	16.9	19.7	30.5	—	3.3

* Initial: the tumor cells separated from the tumor bearing mice at the start of the experiment. Control: the tumor cells separated from the tumor bearing mice starved for 6 hours. Irradiated: the tumor cells separated from the tumor bearing mice starved for 6 hours after whole body irradiation.

Table 5 Effect of X-irradiation on the composition of total fatty acids of livers of the tumor bearing mice

Experiment Group*		Percentage Composition						
		$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:2'}$	$C_{20:poly.}$
1	Initial	24.8	4.2	14.6	17.2	25.5	3.7	8.6
	Control	24.3	3.2	18.0	15.2	25.5	—	13.8
	Irradiated	27.3	3.5	16.9	15.8	23.8	1.5	11.1
2	Control	32.5	2.6	20.2	16.7	22.2	—	5.8
	Irradiated	32.5	3.3	19.2	19.5	22.2	—	3.7

* Initial: the livers separated from the tumor bearing mice at the start of the experiment. Control: the livers separated from the tumor bearing mice starved for 6 hours. Irradiated: the livers separated from the tumor bearing mice starved for 6 hours after whole body irradiation.

with the changes of the fatty acid composition of liver which is recognized as a most important organ in fatty acid metabolism of animals. As shown in Table 5, the proportion of each fatty acid of liver was in descending order of palmitic, linoleic, stearic, oleic, eicosa-polyenoic and palmitoleic acids. No remarkable changes were observed in the fatty acid composition of the liver with whole body irradiation, however, the proportion of the unsaturated fatty acids to saturated ones decreased by approximately 10 per cent more than that of controls.

DISCUSSION

It is well known that swollen mitochondria induced by swelling agents usually show a reduced activity of oxidative phosphorylation. The lipid fraction prepared from the ascitic fluid of tumor bearing mice after X-irradiation induced swelling of rat liver mitochondria and also uncoupled the oxidative phosphrylation of the tumor cells[1]. The swelling action of non-esterified fatty acids is in

proportion to the uncoupling activity[4] or to the stimulating activity on the latent ATPase[5], and the activities could be reduced by the esterification[6,7] or by the formation of albuminate[4,8-10]. Furthermore, the uncoupling activity or swelling action of non-esterified fatty acids is far more active in unsaturated acids[4] than in the corresponding saturated acids.

In this experiment, there were no significant differences in the lipid contents between the ascitic fluids obtained from the control and X-irradiated groups. On this respect, ELKO *et al.* [11] also obtained similar results, namely, the content of triglyceride and non-esterified fatty acids in plasma of rabbits did not change significantly 6 hours after whole body irradiation (1000 r). The proportion of unsaturated fatty acids, however, increased remarkably in the total and non-esterified fatty acids obtained from the ascitic fluid of the X-irradiated group. Therefore, the main reason for displaying the potent activity as a swelling or an uncoupling agent in the ascitic fluid of the X-irradiated mice can be ascribed to a much greater proportion of unsaturated acids, especially oleic and linoleic acids.

With respect to the increment of unsaturated fatty acids in the ascitic fluid by the X-irradiation, nothing is yet known about the mechanism which may involve enzyme reactions concerned with unsaturation of fatty acids or hydrolysis of precursor lipids. The following two possibilities[12], however, can be considered: One of them is a stimulation of hydrolytic split of unsaturated fatty acids from compound lipids and the other is a stimulation of unsaturation of fatty acids. The former possibility is supported by the increment of lysophosphatides with X-irradiation[13]. The latter one is supported by the fact that the proportion of unsaturated fatty acids in ascitic fluid increased considerably without remarkable decrease in that of liver after X-irradiation. Futhermore, this may be supported by the fact[14] that *de novo* synthesis of fatty acids, especially unsaturated fatty acids, in rabbit bone marrow homogenates was remarkably stimulated immediately after whole body irradiation (840 r). The increment of unsaturated acids, especially oleic and linoleic acids, in the total and non-esterified fatty acids of ascitic fluid, would be ascribed to fatty acids released into the ascitic fluid from liver or other organs by whole body irradiation.

On the other hand, several endogenous uncoupling agents were isolated from various subcellular particles such as rat liver microsome[5,15], aged and swollen mitochondria[6,9,10] and insect sarcosome[16], and were clarified that all of these uncoupling agents contained non-esterified, long-chain fatty acids. The endogenous uncoupling agent would act as a factor for the regulation of energy metabolism in the cells. The uncoupling agent as indicated in this paper also would play an important role in the regulation mechanism of oxidative phosphorylation of the tumor cells.

There are many reports on the inhibitory effect of X-ray on oxidative phosphorylation in various animals, however, under rigidly controlled experimental conditions, oxidative phosphorylation of rat liver mitochondria fall to approximately 75 per cent at 6 hours and return to the normal level at 24 hours after whole body irradiation (840 r)[17]. Although the mechanism of this uncoupling of oxidative phosphorylation is yet obscure, it may be similar to that in the tumor cells described in this paper.

Attempts are being made to elucidate the mechanism of uncoupling oxidative phosphorylation in mouse liver mitochondria after whole body irradiation in relation to this problem.

SUMMARY

It was investigated to clarify the relationship between the composition of the lipid fractions obtained from the ascitic fluid of Ehrlich ascites tumor bearing mice and its uncoupling activity after whole body irradiation (1,000 r).

1. Oxidative phosphorylation of Ehrlich ascites tumor cells was loosely uncoupled with the addition of ascitic lipid fraction extracted from tumor bearing mice.

2. The uncoupling activity of the lipid fraction on the oxidative phosphorylation of the tumor cells increased after whole body irradiation.

3. Ascitic lipid fraction, especially acetone soluble fraction accelerated mitochondrial swelling, and the swelling action was increased remarkably by the whole body irradiation.

4. No significant changes were observed in the proportion of acetone soluble fraction to acetone insoluble fraction in the ascitic lipid after X-irradiation, and the proportion of the both fractions was approximately 9 : 1, respectively.

5, Main compositions of total and non-esterified fatty acids in the ascitic fluid obtained from the control and X-irradiated groups were palmitic, stearic, oleic, linoleic and palmitoleic acids, and the proportions of unsaturated acids, especially oleic and linoleic acids in both fatty acid fractions were greater in the X-irradiated group.

6. Remarkable increment of unsaturated fatty acid, especially linoleic acid, was also observed in the total fatty acids of the tumor cells separated from the X-irradiated group.

7. It can be concluded that an uncoupling agent extracted from ascitic fluid of the X-irradiated group was a mixture of long-chain fatty acids, especially oleic and linoleic acids.

8. It was also discussed that uncoupling oxidative phosphorylation in liver mitochondria after whole body irradiation may be caused by a similar mechanism

to that in the tumor cells.

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