RELATION BETWEEN CARDIOTOXIC EFFECT OF ADRIAMYCIN AND SUPEROXIDE ANION RADICAL*

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The investigation was undertaken to study a possible mechanism for adriamycin cardiotoxicity. The activities of superoxide dismutase and catalase in the heart of mice were increased significantly by the intraperitoneal administration of 15 mg/kg of adriamycin. In contrast, these enzymes in the liver and kidney were unaffected by this dose of adriamycin.

In vitro studies revealed that adriamycin inhibited the NADH-cytochrome c oxidoreductase activity of mitochondria in the guinea pigs heart. Moreover adriamycin stimulated the formation of superoxide anion radical in mitochondria isolated from guinea pigs. Particularly, the formation of superoxide anion radical in the heart mitochondria was 5 times higher than that in the liver mitochondria particle. On the other hand, the contents of superoxide dismutase in the heart were significantly lower than that in the liver. These results suggest that the cardiotoxic effect of adriamycin is caused by the following mechanism: adriamycin directly stimulates the formation of superoxide anion radical, particularly in the heart mitochondria. In spite of the induction of defence enzymes such as superoxide dismutase and catalase, their abilities seem to be swamped by enhanced active oxygen radicals.

Keywords—adriamycin; cardiotoxicity; superoxide anion radical; superoxide dismutase; catalase

INTRODUCTION

Adriamycin, an anthracycline antibiotic, possesses a high chemotherapeutic effect against acute leukemia and various solid neoplasms. However, the clinical usefulness of this antibiotic has been limited by its undesirable cardiotoxic side effect.

Endocardial biopsies showed marked pathological alterations of the heart tissue after administration, for example, myofibrillar fragmentation, mitochondrial disruption and glycogen depletion.^{1,2)} In mice, intraperitoneal injection of 15 mg/kg of adriamycin induced these alteration in heart tissue.²⁾ Moreover, changes in electrocardiograms were also observed.^{1,3)}

Several hypothesis have been proposed to explain the mechanism of cardiotoxicity caused by

adriamycin: (1) the inhibition of DNA and/or RNA syntheses;⁴⁾ (2) the inhibition of enzyme activity in the mitochondrial respiratory chain;^{5,6)} (3) the increase of intracellular calcium concentration;⁷⁾ (4) the inhibition of Na-K ATPase;⁸⁾ and (5) peroxidation of cardiac lipids^{2,9)} but the precise mechanism remains unknown.

The incidence of this side effect increases significantly when the cumulative dose exceeds 550 mg/m² body surface area¹⁰⁾ and dose limitation has been the easiest way to prevent this myopathy. Recently, an approach has been undertaken with the combined administration of some other drugs, such as vitamin E (α -tocopherol)¹¹⁾ and vitamin C (ascorbate).¹¹⁾ These drugs are potent antioxidants, and may prevent lipid peroxidation.

It is well known that the lipid peroxidation was initiated by active molecular oxygen, such as

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superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and singlet oxygen (1O_2). In these species, hydrogen peroxide, hydroxyl radical and singlet oxygen are second oxygen species of superoxide anion radical. Superoxide dismutase (SOD), a scavenger of superoxide anion radical, prevents this lipid peroxidation.¹²⁾

In this connection, we have investigated the effects of adriamycin on oxygen adaptive enzymes, lipid peroxidation and superoxide anion radical formation, and discussed the relation between superoxide and cardiotoxicity induced by adriamycin.

MATERIALS AND METHODS

Materials — Adriamycin hydrochloride was obtained from Kyowa Hakko Kogyo Co., Ltd. (Japan). Cytochrome c (Type III), xanthine and peroxidase (Type VII) were purchased from Sigma Chemical Co., Ltd. (U.S.A.), adrenaline bitartrate and malondialdehyde bis (MDA) were from Tokyo Chemical Industry Co., Ltd. (Japan) and NADH was from Oriental Yeast Co., Ltd. (Japan). Xanthine oxidase was purified from bovine milk by the method of Nathans and Kirby¹³⁾ and deflavo xanthine oxidase was prepared by the method of Komai *et al.*¹⁴⁾ with a minor modification. Human Cu,Zn-SOD was purified from erythrocyte by the method of McCord and Fridovich.¹⁵⁾ Mouse Cu,Zn-SOD was purified from liver by the method of Reiss and Gershon.¹⁶⁾ All other chemicals were of the highest purity available.

Experimental Animals — Male Hartley guinea pigs and male ddY mice were obtained from Shizuoka Agricultural Co. Association of Laboratory Animals (Japan). The animals were provided with a standard laboratory diet and water.

Tissue Handling for Enzymatic Analysis — Male ddY mice (about 20 g of body weight) were treated intraperitoneally with 15 mg/kg of adriamycin dissolved in 0.9% NaCl solution. At various time intervals after the injection, the mice were killed by decapitation. Each group consisted

of 5 mice. The heart, liver and kidney excised were collected from 5 mice, minced and homogenized in 4-vol. of ice-cold 1.15% KCl solution by Polytron. Cell debris and nuclei were separated by centrifugation at $600 \times g$ for 15 min. Aliquots of the supernatant were quickly frozen at $-20^\circ C$ and stored until the analysis. Protein was determined by the method of Lowry *et al.*¹⁷⁾

Preparation of Mitochondria Particle from Guinea Pigs — Male Hartley guinea pigs (about 250 g of body weight) were treated intraperitoneally with 5 mg/kg of adriamycin dissolved in 0.9% NaCl solution. Twenty-four hours after the injection, the guinea pigs were killed by decapitation. The heart and liver were excised and mitochondria particles were prepared. Preparation of heart mitochondria was performed by the method of Tyler and Gonze.¹⁸⁾ Liver mitochondria was prepared according to the method of Hogeboom,¹⁹⁾ with a minor modification.

Assay of Enzyme Activity — SOD activity was measured by the method of McCord and Fridovich.¹⁵⁾ Under the defined conditions, one unit of the activity is defined as the amount of SOD required to inhibit the rate of reduction of cytochrome c by 50%. Catalase activity was measured by the method of Bergmeyer.²⁰⁾ The change in optical density of hydrogen peroxide at 240 nm was measured. Assay of Cu,Zn-SOD content was performed by the rocket immunoelectrophoresis according to the previous paper.²¹⁾ Mitochondria NADH-cytochrome c oxidoreductase activity was assayed by the method of Green and Ziegler.²²⁾

Assay of Lipid Hydroperoxide Content — Lipid hydroperoxide concentration was measured by the thiobarbituric acid assay²³⁾ and iodometric assay.²⁴⁾

Lipid Analysis — Extraction of membrane lipid and analysis of phospholipid and fatty acid were performed by the method of Colbeau *et al.*²⁵⁾ Lipid phosphorus was determined by the method of Bartlett.²⁶⁾

Determination of Superoxide Anion Radical — Superoxide anion radical formation was determined by the cooxidation of epinephrine to

adrenochrome according to the method of Thayer.²⁷⁾

RESULTS

1. Effect of Adriamycin on Superoxide Dismutase and Catalase

Activities of oxygen adaptive enzymes in mice injected intraperitoneally with 15 mg/kg of adriamycin were assayed. As shown in Fig. 1, cardiac SOD activity gradually increased during the 72-h period after administration, and the increase became significant after 48 h ($121 \pm 8.5\%$, percent of the preinjection activity $\pm$ S.D.) and 72 h ($127 \pm 4.5\%$). In contrast to the change of cardiac SOD, no significant change in SOD was detected in the liver and kidney. Moreover, the change of Cu,Zn-SOD protein content in homogenate was investigated by rocket immunoelectrophoresis and compared with Cu,Zn-SOD activity. Cu,Zn-

SOD protein content showed the same behaviour with its activity and the correlation of coefficient between these two values was 0.997. This result indicates that the induced SOD is the active type (Table I).

Catalase activity in the heart also increased significantly and reached $189 \pm 23\%$ at 48 h and $230 \pm 24\%$ at 72 h after the administration, whereas hepatic and renal catalase activity showed no significant change (Fig. 2).

It was assumed that these protective enzymes had been induced as a defence system against superoxide increased by the injection of adriamycin.

2. Effect of Adriamycin on Lipid Peroxidation

It has been discussed that lipid peroxidation of cardiac cell membrane may play an important role in cardiotoxicity induced by adriamycin.³⁾ Therefore, lipid peroxide concentration was

FIG. 1. Superoxide Dismutase Activities after the Administration of Adriamycin

Details of experimental design are given in Materials and Methods. Data is mean $\pm$ S.D. of 4 determinations.

Key: superoxide dismutase activity in heart (●), liver (○) and kidney (▲); a): significant difference between pre- and post-administration level ($p < 0.05$).

FIG. 2. Catalase Activities after the Administration of Adriamycin

Details of experimental design are given in Materials and Methods. Data is mean $\pm$ S.D. of 4 determinations.

Key: catalase activity in heart (●), liver (○) and kidney (▲); a): significant difference between pre- and post-administration level ($p < 0.05$); b): $p < 0.01$.

assayed by the iodometric method and the thio-barbituric acid method. As shown in Fig. 3 and 4, lipid peroxide level in liver homogenate increased significantly, but the increase of lipid hydroperoxide level in the heart and kidney were not observed in the both methods. The result that adriamycin administration induced a significant increase in lipid peroxide levels in liver, but not in heart, is similar to that of the previous report.¹¹⁾ These results suggest that the cardiotoxicity by adriamycin is not only due to production of lipid peroxide. But we are unable to explain why the lipid peroxide level in the liver increased. Moreover, the phospholipid pattern and fatty acid composition of phospholipid in homogenate of heart, liver and kidney from adriamycin administered mice and control animals were com-

pared. But no significant change could be observed (Table II and III).

3. Effect of Adriamycin on the Respiratory Chain of Mitochondria

In order to investigate whether adriamycin itself could function as an electron acceptor, adriamycin was incubated in a medium containing xanthine and xanthine oxidase. Under these conditions, decrease of absorbance at 490 nm, which is characteristic of adriamycin, occurred (Fig. 5); but decrease of absorbance was not observed when deflavo xanthine oxidase was used instead of xanthine oxidase. These results suggested that adriamycin was able to undergo enzymatic single-electron reduction by xanthine oxidase. This single electron is assumed to be transferred from FAD in xanthine oxidase,

TABLE I. Effect of Adriamycin on Superoxide Dismutase

Organ	Hours after treatment	Cu,Zn-SOD activity (U/mg of protein)	
		Cytochrome c method	Rocket immuno-electrophoresis ^{a)}
Heart	0	2.30	2.29
	6	2.30	2.65
	12	2.48	2.58
	24	2.44	2.51
	48	2.63	2.66
	72	3.08	2.86
Liver	0	14.9	15.4
	6	15.5	14.8
	12	15.1	14.6
	24	15.6	15.3
	48	14.8	13.8
	72	13.2	13.7
Kidney	0	7.38	7.48
	6	7.92	8.25
	12	6.76	7.07
	24	7.65	7.55
	48	9.27	9.21
	72	9.16	9.06

a) Values are expressed as activities calculated from the protein contents determined by rocket immuno-electrophoresis and the relative activity of SOD (3200 U/mg).

because in the case of deflavo xanthine oxidase, which has no FAD, adriamycin could not be reduced. As adriamycin has a quinone structure similar to coenzyme Q_{10} in the respiratory chain, it possibly inhibited the electron-transport by competing with coenzyme Q_{10} . This postulate was confirmed by the fact that mitochondrial NADH-cytochrome c oxidoreductase activity was apparently inhibited by adriamycin (Fig. 6).

4. Effect of Adriamycin on Superoxide Formation

Quinones can undergo enzymatic single-electron reduction to the semiquinone radical, which can transfer one electron to molecular oxygen and form the superoxide anion radical,²⁸⁾ and it is known that adriamycin stimulates the formation of superoxide anion radical in the sub-mitochondrial particle.²⁹⁾ From a hypothesis that the stimulation of superoxide formation by

adriamycin is an important factor in regard to cardiotoxicity, we compared the production of superoxide anion radical by cardiac mitochondria and hepatic mitochondria from guinea pigs in the presence of adriamycin. Oxidation of NADH by mitochondrial particle in the presence of oxygen caused by the conversion of epinephrine to adrenochrome, measured by absorbance at 485 nm. In the absence of adriamycin, the absorbance at 485 nm scarcely changed. The addition of increasing concentrations of adriamycin progressively stimulated the formation of adrenochrome, and SOD inhibited this reaction. This result suggested that adrenochrome formation was due to superoxide anion radical. The formation of superoxide anion radical by cardiac mitochondria was 5 times higher than that by hepatic mitochondria (Fig. 7). The ability to pro-

FIG. 3. Lipid Hydroperoxide Levels after the Administration of Adriamycin

Details of experimental design are given in Materials and Methods. Data is mean $\pm$ S.D. of 3 determinations.

Key: lipid hydroperoxide level in heart (●), liver (○) and kidney (▲); a): significant difference between pre- and post-administration level ($p < 0.05$); b): $p < 0.01$.

FIG. 4. Malondialdehyde Levels after the Administration of Adriamycin

Details of experimental design are given in Materials and Methods. Data is mean $\pm$ S.D. of 3 determinations.

Key: malondialdehyde level in heart (●), liver (○) and kidney (▲); a): significant difference between pre- and post-administration level ($p < 0.05$).

duce superoxide anion radical per NADH-cytochrome c oxidoreductase activity of cardiac mitochondria was also 5 times more than hepatic mitochondria. This difference of ability to produce superoxide anion radical by each mitochondria was not due to the SOD content in mitochondria, because the hepatic mitochondria particle used in this experiment contained SOD only 1.5-fold more than in cardiac mitochondria particle. From these results, it was assumed that

TABLE II. *Phospholipid Pattern in Homogenate from Mice administrated of Adriamycin*

Phospholipid	Heart		Liver		Kidney	
	Control	Adriamycin treated	Control	Adriamycin treated	Control	Adriamycin treated
Cardiolipin	5.9	5.6	3.0	2.4	3.5	2.9
Phosphatidylcholine	50.9	49.6	57.7	55.6	41.9	47.8
Phosphatidylethanolamine	38.2	38.0	27.5	30.1	39.0	33.2
Phosphatidylinositol	2.9	4.5	7.4	7.7	5.9	4.6
Phosphatidylserine	2.1	2.2	4.2	3.9	3.5	2.9

The data represent per cent values of total lipids.

TABLE III. *Fatty Acid Composition of Phospholipids in Homogenate from Mice administrated of Adriamycin*

Phospholipid	Fatty acid	Heart		Liver		Kidney	
		Control	Adriamycin treated	Control	Adriamycin treated	Control	Adriamycin treated
Phosphatidylethanolamine	Palmitic acid	19.9	13.4	31.3	31.3	22.0	18.0
	Stearic acid	49.3	53.1	38.5	38.8	47.7	47.1
	Oleic acid	14.7	18.6	17.6	13.7	14.2	17.3
	Linoleic acid	16.0	15.0	12.6	16.1	16.1	16.7
Phosphatidylcholine	Palmitic acid	31.1	36.6	46.5	47.8	44.5	42.7
	Stearic acid	33.2	37.4	35.3	35.5	22.0	22.2
	Oleic acid	23.3	15.5	10.4	7.5	21.5	18.9
	Linoleic acid	12.5	10.7	7.9	9.1	15.1	16.4

Values are expressed as percentage of recording area of the total peak areas of fatty acids in gas liquid chromatography.

this difference in the ability to produce superoxide anion radical was the most important factor, and the toxicity of adriamycin appeared particularly in the heart. However, we could not observe the increase of malondialdehyde, when mitochondria was incubated with adriamycin.

5. Effect of Adriamycin on Mitochondrial Lipid Hydroperoxide Formation

We investigated the effect of adriamycin on the formation of lipid hydroperoxide, using mitochondria particle from guinea pigs. It was compared with the malondialdehyde content in mitochondria prepared before injection and 24 h after the intraperitoneal administration of 5 mg/kg adriamycin. The concentration of malondialdehyde increased 1.9-fold ($1.61 \pm 0.26 \rightarrow 3.00 \pm 0.49$ nmol MDA/mg of protein, $n = 3$, significant difference between pre- and post-ad-

ministration level, $p < 0.05$) in heart mitochondria and 2.7-fold ($1.21 \pm 0.24 \rightarrow 3.26 \pm 0.60$ nmol MDA/mg of protein, $n = 3$, significant difference between pre- and post-administration level, $p < 0.05$) in liver mitochondria by the injection of adriamycin.

DISCUSSION

It has been shown that intact mitochondria can be made to produce superoxide anion radical even in normal condition. Most of the superoxide anion radical can be scavenged by the protective enzymes such as SOD, catalase and glutathione peroxidase. When adriamycin is injected, it stimulates the production of superoxide by the mitochondria particle. Especially in the presence of adriamycin, the formation of superoxide anion radical by heart mitochondria was several times

FIG. 5. Changes of Absorption Spectra of Adriamycin by Xanthine-Xanthine Oxidase System

Two and three-fifths ml of medium containing 0.1 M phosphate buffer (pH 8.5), 0.22 mM xanthine and $26 \mu\text{M}$ of adriamycin was preincubated at 37°C for 5 min. After the addition of $50 \mu\text{l}$ of xanthine oxidase (XOD, 0.24U/mg) solution (0–0.5 U/ml), the mixture was incubated at 37°C for 20 min. A half ml of 0.1 N HCl solution subsequently added and the spectrum was recorded.

FIG. 6. Effect of Adriamycin on Mitochondrial NADH-cytochrome c Oxydoreductase Activity

Two and half ml of medium containing 20 mM Na-HEPES buffer (pH 7.5), 0.25 M sucrose and indicated concentrations of adriamycin was preincubated at 25°C for 5 min. After the addition of 0.1 ml of 0.3 M ferricytochrome c solution, $10 \mu\text{l}$ of mitochondria fraction and 0.1 ml 10 mM NADH solution, the mixture was incubated at 25°C and the change of absorbance at 550 nm was measured.

FIG. 7. *Effect of Adriamycin on Superoxide Anion Radical Formation in Mitochondria*

The change of absorbance at 485 nm was recorded in 3 ml of aerobic medium containing 0.25 M sucrose, 20 mM Na-HEPES buffer (pH 7.5), 1 mM adrenaline bitartrate, 0.4 mM NADH, indicated concentration of adriamycin and 0.126 mg of heart mitochondria fraction (●) or 0.129 mg of liver mitochondria fraction (○) or 0.126 mg of heart mitochondria fraction and 30 units of superoxide dismutase (×).

higher than that by liver mitochondria. On the other hand, the capacity to detoxify reactive oxygen radicals, in other words, the content of SOD and catalase in the heart is lower than that in the liver.

Injection of adriamycin induced increases of SOD and catalase contents, the enzymes protecting mitochondria from deleterious effects of reactive oxygen species. SOD activity increased only 27% after the administration. However, this small increase of SOD may suggest the existence of superoxide anion radical above normal concentrations.

The finding that the increase of catalase activity is 5 times higher than that of SOD activity is consistent with the previous report concerning the changes of these enzymes under hyperoxic conditions.³⁹⁾ The increase of catalase activity may indicate the increase of hydrogen peroxide which takes part in the formation of highly reactive hydroxyl radical together with superoxide anion radical. The increase of both enzymes activities may be a defence system against oxygen toxicity, and the coordinated increase of these protective enzymes may be required for maximum resistance to damage by reactive oxygens.

FIG. 8. *Possible Mechanism of Adriamycin Cardiotoxicity and Defense System against Reduced Form of Oxygen*

In spite of the induction of these defence enzymes, the ability of enzymes seems to be swamped by enhanced oxygen radicals.

In conclusion, a possible mechanism of adriamycin stimulated cardiotoxicity is shown in Fig. 8. Adriamycin may function as an artificial chain. Non-enzymatic oxidation of a reduced form of adriamycin by molecular oxygen will then produce superoxide anion radical. This superoxide is converted to hydrogen peroxide. Superoxide itself or hydroxyl radical, which is formed from superoxide anion radical and hydrogen peroxide, can trigger free radical reaction leading to the peroxidation of poly-unsaturated fatty acid components of membrane lipids and inhibition of respiratory chain.

However, the production of superoxide anion radical or the participation of flavoprotein in cytoplasm cannot be neglected. We consider that superoxide production on mitochondria is most important factor of cardiotoxicity by adriamycin, because adriamycin inhibits electron transport in mitochondria competing with coenzyme Q_{10} . Moreover lipid peroxide produced by superoxide uncouples the respiratory chain and inhibits the change of ADP to ATP which is necessary to preserve of dynamic function of heart.

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