LIPID PEROXIDATION BY BLEOMYCIN-IRON COMPLEXES *IN VITRO*

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Lipid peroxidation catalyzed by bleomycin (BLM)-metal complexes was studied *in vitro* using arachidonic acid as the substrate. Iron complexes of BLM caused extensive lipid peroxidation, but other metal complexes did not. The lipid peroxidation caused by the iron complexes was inhibited by antioxidants such as *dl-α*-tocopherol, ascorbic acid *etc.*, but not by other scavengers of hydroxyl and superoxide radicals, and of singlet oxygen. Cyanide ion suppressed the lipid peroxidation caused by BLM-Fe(II), but did not suppress the peroxidation activity of BLM-Fe(III). The peroxidation activity of BLM-Fe(II) was lost instantly by pre-incubation of the complex at 37°C before mixing with arachidonic acid, but that of BLM-Fe(III) was not. These results indicate that the active form for the lipid peroxidation derived from BLM-Fe(II) differs from that of BLM-Fe(III).

Bleomycin (BLM) has been clinically used as an important cancer chemotherapeutic agent for the treatment of several types of malignancies[1]. In order to enhance the therapeutic effect, the mechanism of pulmonary toxicity[2] or BLM analogues having less toxicity has been studied. It has been reported that pulmonary toxicity due to BLM was enhanced when patients were exposed to a high concentration of oxygen[3]. This was confirmed by animal experiments using hamsters[4]. We observed the time-dependency of the oxygen effect on BLM-induced pulmonary toxicity and reported that (a) when mice were exposed to a high concentration of oxygen during or after BLM treatment the pulmonary toxicity was increased and (b) the toxicity was reduced when animals were exposed before the BLM treatment[5]. We also reported that scavengers of active oxygen species and peroxy radicals such as *dl-α*-tocopherol[6], ascorbic acid and glutathione[7] suppressed the pulmonary toxicity of BLM in mice. The active form of BLM for DNA cleavage appears to be the ternary complex: BLM-Fe(III)-O_2H^- [8]. These findings suggest that "active oxygen" species formed from BLM-iron complexes are involved in the BLM-induced pulmonary toxicity.

In this paper we report on lipid peroxidation catalyzed by BLM-iron complexes *in vitro*, which may be related to the pulmonary toxicity caused by BLM.

Materials and Methods

Chemicals

BLM A_2, which is the major component of clinically used BLM, was used in the experiments. The Fe(II) and Fe(III) complexes of BLM A_2 were prepared by addition of an equimolar amount of ferrous sulfate or ferric chloride to a BLM A_2 aqueous solution at 0°C just before use. Other metal complexes were prepared by a similar procedure. Arachidonic acid (ARA) and 3-(2-pyridyl)-5,6-bis-

Table 1. Lipid peroxidation catalyzed by BLM A_2-metal complexes.

Metal	Fe^{2+}		Fe^{3+}		Co^{2+}		Ni^{2+}		Cu^{2+}		Zn^{2+}	
BLM A_2	+[a]	−[b]	+	−	+	−	+	−	+	−	+	−
Absorbance (A_{532})	0.83	0.26	0.42	0.10	0.24	0.16	0.06	0.03	0.02	0.03	0.03	0.04

[a] With BLM A_2, [b] without BLM A_2.

Fig. 1. Absorption spectra of the adducts of TBA and the products derived from lipid peroxides formed by reaction of ARA and BLM A_2-iron complexes.

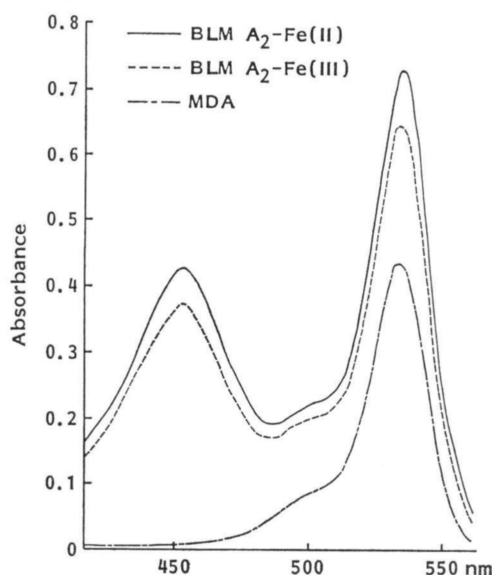
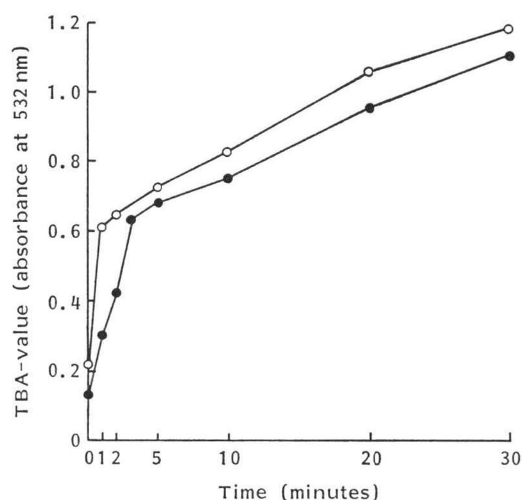


Fig. 2. Time course of lipid peroxidation catalyzed by BLM A_2-iron complexes.
○ BLM A_2-Fe(II), ● BLM A_2-Fe(III).



(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine) were purchased from Sigma Chemical Co., USA, and thiobarbituric acid (TBA) from E. Merck, West Germany. All other chemicals were of reagent grade.

Determination of Lipid Peroxidation *In Vitro*

Malondialdehyde (MDA) formed by acidic treatment of peroxidated ARA was measured by colorimetry after reaction with TBA. The reaction mixture for lipid peroxidation contains 0.68 mM BLM-metal complex, 10 mM ARA with or without other substances such as scavengers in 0.4 ml of Krebs-Ringer phosphate buffer (pH 7.3). The mixture was incubated at 37°C for 5 minutes. In order to terminate the reaction, 0.02 ml of 50% trichloroacetic acid was added at 0°C. After addition of 0.2 ml of 1% TBA, the solution was incubated at 37°C for 30 minutes. Then, 1 ml of butanol was added, and the mixture was vigorously shaken and centrifuged at $700\times g$ for 10 minutes. The absorbance of the butanol layer at 532 nm was measured by spectrophotometry. 1,1,3,3-Tetraethoxypropane, the acetal of MDA, was used to prepare the calibration curve for MDA (Fig. 1).

Determination of Fe(II) Ion

Fe(II) ion formed during lipid peroxidation by BLM-Fe(III) was measured by the absorbance of Ferrozine-Fe(II) complex at 565 nm.

Results

The effect of various BLM-metal complexes on lipid peroxidation of ARA was examined first. As shown in Table 1, BLM-Fe(II) and -Fe(III) enhanced the lipid peroxidation markedly, but other

Table 2. Effect of radical scavengers on lipid peroxidation catalyzed by BLM A_2-iron complexes.

	Scavenger	Radical	TBA value (A_{532})	Inhibition (%)
BLM A_2-Fe(II)	—[a]		0.75	0.0
	dl-α-Tocopherol	$O_2^{-\cdot}$, $^1O_2$, ROOH[b]	0.07	90.8
	Superoxide dismutase	$O_2^{-\cdot}$	0.66	11.9
	D-(−)-Mannitol	·OH	0.68	9.9
	Benzoic acid		0.69	8.9
	Dimethoxyethane		0.69	8.9
	β-Calotene	$^1O_2$	0.76	0.0
	Guanosine		0.82	−8.9
	2,5-Dimethylfuran		0.77	−1.6
	Catalase	H_2O_2	0.38	50.3
	Cysteine	ROOH	0.21	72.7
	Glutathione		0.48	36.6
	Ascorbic acid		0.43	68.4
	NADPH		0.43	42.6
BLM A_2-Fe(III)	—		0.52	0.0
	dl-α-Tocopherol	$O_2^{-\cdot}$, $^1O_2$, ROOH	0.08	85.0
	Superoxide dismutase	$O_2^{-\cdot}$	0.50	4.8
	D-(−)-Mannitol	·OH	0.50	4.8
	Benzoic acid		0.69	−32.7
	Dimethoxyethane		0.47	10.0
	β-Calotene	$^1O_2$	0.54	−2.9
	Guanosine		0.52	0.0
	2,5-Dimethylfuran		0.49	5.8
	Catalase	H_2O_2	0.27	48.8
	Cysteine	ROOH	0.15	72.2
	Glutathione		0.39	26.5
	Ascorbic acid		0.29	44.7
	NADPH		0.23	56.7

[a] Control, [b] ROOH indicates lipid peroxides.

metal complexes did not. The same result was also obtained when linolenic acid was used as the substrate (data are not shown). The lipid peroxidation in the presence of the BLM-iron complexes proceeded biphasically (Fig. 2). The initial rapid phase lasted about 1 and 3 minutes for BLM-Fe(II) and BLM-Fe(III), respectively.

The effect of various radical scavengers on the lipid peroxidation catalyzed by BLM-iron complexes was examined next. The result is shown in Table 2. *dl*-α-Tocopherol, which scavenges superoxide and peroxy radicals as well as singlet oxygen, showed the most effective antioxidant activity. Other antioxidants such as ascorbic acid, cysteine *etc.* also inhibited the lipid peroxidation. Native catalase as well as the heat-inactivated enzyme showed inhibitory activity (data are not shown). Superoxide dismutase, $^1O_2$-scavengers such as β-calotene, guanosine *etc.* and ·OH-scavengers such as D-mannitol, benzoic acid *etc.* did not inhibit the lipid peroxidation.

Table 3. Effects of cyanide and azide ions on lipid peroxidation catalyzed by BLM A_2-iron complexes.

	Ion	TBA value (A_{532})	Inhibition (%)
BLM A_2-Fe (II)	—[a]	0.85	0.0
	CN^-	0.21	75.7
	N_3^-	0.84	1.2
BLM A_2-Fe (III)	—	0.63	0.0
	CN^-	0.59	6.4
	N_3^-	0.74	−16.5

[a] Control.

The effect of cyanide and azide ions on the lipid peroxidation of BLM-iron complexes was ex-

Fig. 3. Absorption spectra of BLM A_2-Fe(II) and -Fe(III) ligated with cyanide and azide ions.

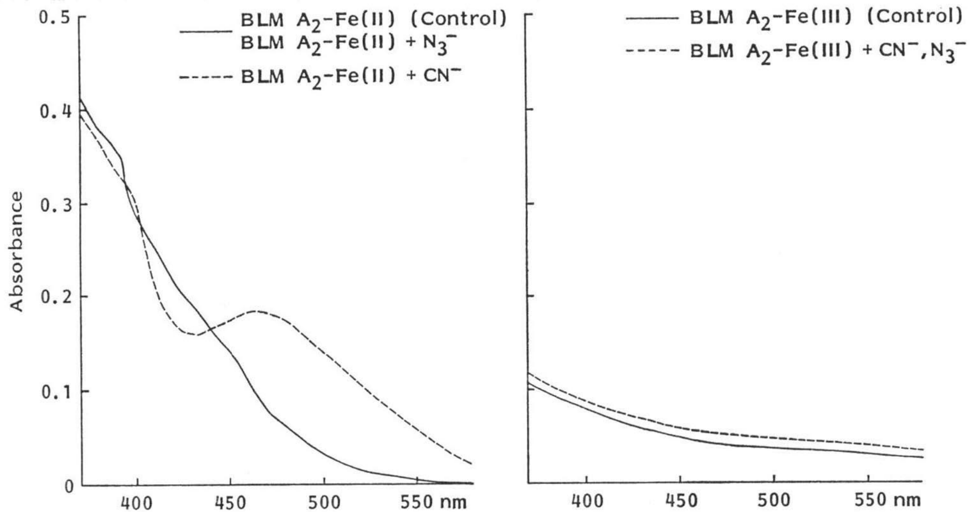
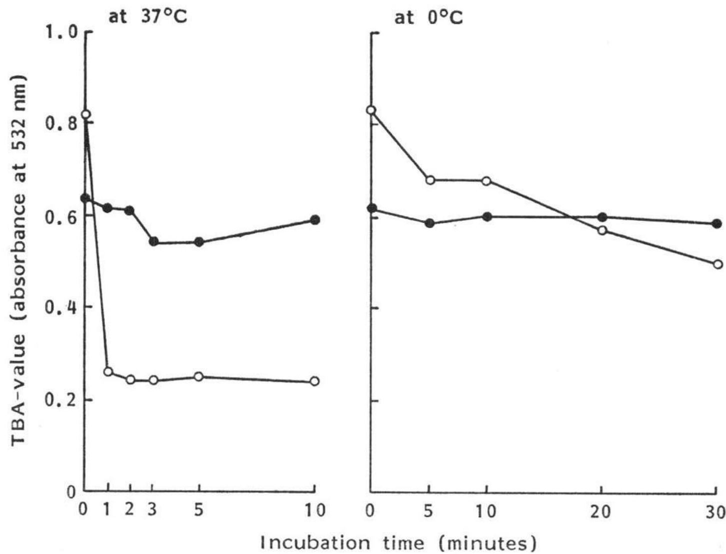


Fig. 4. Stability of lipid peroxidation activities of BLM A_2-iron complexes.
○ BLM A_2-Fe(II), ● BLM A_2-Fe(III).



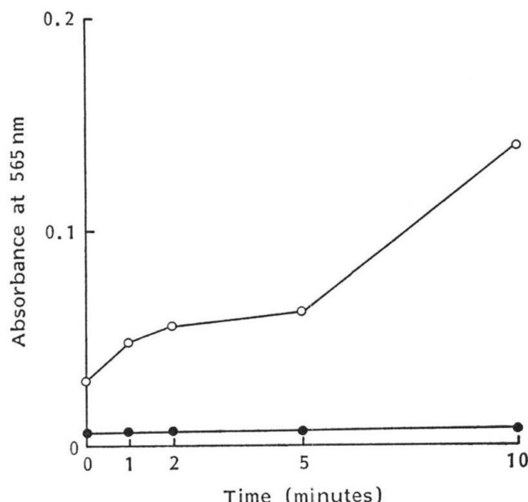
amined, because these ions seem to compete for the coordination site of oxygen in BLM-iron complexes. The lipid peroxidation activity of BLM-Fe(II) was inhibited by cyanide ion (1 mM), but that of BLM-Fe(III) was not (Table 3). Azide ion did not inhibit the lipid peroxidation of either BLM-Fe(II) or -Fe(III). The visible spectra showed that the spectrum of BLM-Fe(II) changed distinctly upon the addition of cyanide ion (Fig. 3).

The stability of the lipid peroxidation activities of BLM-iron complexes was examined. As shown in Fig. 4, the peroxidation activity of BLM-Fe(II) was lost instantly upon pre-incubation at 37°C, but at 0°C it gradually decreased with the incubation time ($t_{1/2}$=35 minutes). The lipid peroxidation

Fig. 5. Formation of Fe(II) ion during lipid peroxidation catalyzed by BLM A_2-Fe(III).

(Experimental procedures see Materials and Methods).

○ BLM A_2-Fe(III)+ARA, ● BLM A_2-Fe(III).



activity of BLM-Fe(III) was stable at 37°C and 0°C. The formation of Fe(II) ion was observed during the lipid peroxidation using BLM-Fe(III), but it was not observed when ARA was absent from the reaction mixture (Fig. 5).

Discussion

In the present study, enhancement of lipid peroxidation by BLM-Fe(II) and -Fe(III) complexes (Table 1) and its inhibition by *dl*-α-tocopherol, ascorbic acid and cysteine (Table 2) were demonstrated. As already described, the pulmonary toxicity of BLM was reduced by *dl*-α-tocopherol[6], ascorbic acid and glutathione[7] in mice. Therefore, it is suggested that enhancement of lipid peroxidation by BLM-iron complexes is related to its pulmonary toxicity.

With the exception of *dl*-α-tocopherol, ascorbic acid and cysteine, various radical scavengers did not inhibit the lipid peroxidation activity of BLM-iron complexes (Table 2). Scavengers such as benzoate, mannitol and 2,5-dimethylfuran, which were reported to inhibit lipid peroxidation by active oxygen species generated by a xanthine oxidase system[9,10], did not inhibit the lipid peroxidation activity of BLM-iron complexes. Therefore, the mechanism of peroxidation by BLM-iron complexes seems to be different from that of the xanthine oxidase system. Antioxidants such as *dl*-α-tocopherol, ascorbic acid and cysteine react primarily with a lipid peroxy radical[11]. This might explain the inhibitory effect of these antioxidants on lipid peroxidation activity of BLM-iron complexes.

Inhibition of the lipid peroxidation activity of BLM-Fe(II) by cyanide ion seems to be due to occupation of the coordination site of oxygen by the cyanide ion (Table 3 and Fig. 3).

The instability of the lipid peroxidation activity of BLM-Fe(II) complex, observed when incubated at 37°C in the absence of ARA (Fig. 4), may be due to rapid conversion of the active BLM-Fe(III) complex to an inactive BLM-Fe(III) form as reported by KURAMOCHI *et al.*[8]. However, as described above, a stable BLM-Fe(III) complex prepared directly from BLM and Fe(III) ion also enhanced the lipid peroxidation. The lipid peroxidation activity of this stable BLM-Fe(III) complex was not inhibited by cyanide ion (Table 3) or by pre-incubation in the absence of ARA at 37°C (Fig. 4). The difference between the directly prepared BLM-Fe(III) complex and that made from BLM-Fe(II) was also noticed by POVIRK[12]. An elucidation of the reason for the difference between the two BLM-Fe(III) complexes may contribute to our understanding of the active species of the BLM-iron-complex.

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