ACTIVATION OF BLEOMYCIN-Fe(III) BY BLEOMYCIN-Cu(II) AND CYSTEINE

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When bleomycin (BLM) was incubated with DNA at 37°C for 30 minutes in the presence of BLM-Cu(II) and cysteine, the DNA became more acid-soluble compared with the reaction without BLM-Cu(II). Superoxide dismutase did not suppress this increased DNA chain breakage. This combination of BLM-Cu(II) and cysteine did not enhance BLM-Fe(II)-induced DNA chain breakage, but caused the DNA chain breakage by inactive BLM-Fe(III). Combination of BLM-Fe(III) with BLM-Cu(II) and cysteine also caused production of malondialdehyde-like product from DNA. Anaerobic incubation of DNA with BLM-Fe(III) and BLM-Cu(I) followed by aerobic incubation produced malondialdehyde-like product, but the incubation with CuCl instead of BLM-Cu(I) did not. These results suggest that activation of BLM-Fe(III) by BLM-Cu(II) and cysteine seems to be due to the reduction of BLM-Fe(III) to BLM-Fe(II) by BLM-Cu(I) formed in the reaction of BLM-Cu(II) and cysteine.

Bleomycin (BLM) forms an equimolar complex with various kinds of metal ions such as Cu(II), Fe(II), Fe(III), Zn(II), *etc.*[1]. Among these complexes, BLM-Fe(II) and BLM-Fe(III) with reducing agents cause DNA chain breakage *in vitro*[2~5]. BLM-Cu(II) does not break DNA *in vitro*[6,7], but exhibits antitumor activity[8~12]. We reported that BLM-Cu(II) is transformed to metal-free BLM in cells by reductive liberation of Cu(I), which is trapped by cellular thiol-proteins[13]. In the absence of the Cu(I)-binding proteins, superoxide anion seems to be generated by reaction of Cu(I) with oxygen. We confirmed this fact by reduction of nitroblue tetrazolium chloride in the presence of BLM-Cu(II) and cysteine and by its suppression with superoxide dismutase[14]. Superoxide anion was reported to increase DNA chain breakage activity of BLM[15] and activate inactive BLM-Fe(III)[5]. In the present study, effect of BLM-Cu(II) on DNA chain breakage activity of BLM-iron complexes was examined in the presence of cysteine.

Materials and Methods

Chemicals

Bleomycin A_2 (BLM) and its copper complex (BLM-Cu(II)) were prepared by Nippon Kayaku Co., Ltd., Tokyo. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and $Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$ were purchased from Kanto Chemical Co., Tokyo, CuCl from Wako Pure Chemical Industries, Osaka. BLM-Fe(II) and BLM-Fe(III) were prepared by mixing equimolar aqueous solutions of BLM and the inorganic iron salts at 0°C. BLM-Cu(I) was anaerobically prepared by dissolving powdered CuCl in an equimolar aqueous solution of BLM in an ultrasonic cleaner bath. For the anaerobic preparation, chilled water bubbled with N_2 was used and the N_2 bubbling was kept throughout the preparation.

[^{3}H]DNA was isolated from rat hepatoma AH66F cells cultured with [6-^{3}H]thymidine (5 Ci/mmol, Amersham International plc, Amersham) at 0.15 μCi/ml for 16.5 hours according to MARMUR[16].

Calf thymus DNA was purchased from Sigma Chemical Co., Saint Louis, superoxide dismutase (12,300 units/mg) from Miles Laboratories, Inc., Elkhart, IN, L-cysteine monohydrochloride from Nippon Rikagakuyakuhin Co., Tokyo, and 2-thiobarbituric acid from Nakarai Chemicals, Ltd., Kyoto.

Determination of Acid-solubilization of DNA

After incubating BLM with [^{3}H]DNA, the reaction mixture (0.5 ml) was mixed with 0.1 ml of an aqueous solution containing 10 mg/ml of bovine serum albumin and 0.25 mg/ml of calf thymus DNA, and 0.4 ml of 25% trichloroacetic acid solution containing 25 mM sodium pyrophosphate at 0°C. The mixture was centrifuged at 3,000 rpm for 10 minutes, and the radioactivity contained in 0.5 ml of the supernatant was determined by a liquid scintillation counter.

Determination of Malondialdehyde-like Product from DNA

Malondialdehyde-like product, which was reported to be base-propenals produced from DNA concomitant with DNA chain breakage by BLM[17], was determined according to SAUSVILLE[18]. After reaction of BLM with calf thymus DNA, the reaction mixture (0.2~0.25 ml) was mixed with 2 ml of 0.6% 2-thiobarbituric acid aqueous solution, and incubated for 30 minutes in a boiling water bath. After standing for 5 minutes at room temperature, absorbance at 532 nm was determined by a spectrophotometer.

Results

Table 1 shows enhancement of DNA chain breakage activity of BLM by BLM-Cu(II) in the presence of cysteine and effect of superoxide dismutase on the increased activity. The amount of acid-

Table 1. Effect of BLM-Cu(II) on DNA chain breakage by BLM in the presence of cysteine and influence of superoxide dismutase on the effect of BLM-Cu(II).

Agents	Acid-soluble DNA (% of total DNA)
0.067 mM BLM	16.4
0.065 mM BLM-Cu(II)	5.3
SOD 800 units/ml	0.1
0.067 mM BLM+0.065 mM BLM-Cu(II)	44.8
0.067 mM BLM+0.065 mM BLM-Cu(II)+SOD 800 units/ml	49.5

The mixtures (0.5 ml) containing [^{3}H]DNA (47,600 dpm/μg/ml), 1 mM cysteine, 2 mM KCl and 45 mM potassium phosphate buffer (pH 7.4) with or without 0.067 mM BLM, 0.065 mM BLM-Cu(II) or superoxide dismutase (SOD) of 800 units/ml were incubated for 30 minutes at 37°C. The reaction was terminated and the radioactivity of acid-soluble DNA was counted as described in Materials and Methods. Acidsoluble DNA (%) shown in the table is a mean value of triplicate determinations.

Table 2. Effect of BLM-Cu(II) on DNA chain breakage by BLM-Fe(II) and BLM-Fe(III) in the presence of cysteine.

Agents	Acid-soluble DNA (% of total DNA)
0.0067 mM BLM-Fe(II)	47.0
0.067 mM BLM-Fe(III)	3.9
0.065 mM BLM-Cu(II)	0.0
0.0067 mM BLM-Fe(II)+0.065 mM BLM-Cu(II)	47.2
0.067 mM BLM-Fe(III)+0.065 mM BLM-Cu(II)	48.3

The mixtures (0.5 ml) containing [^{3}H]DNA (62,200 dpm/2 μg/ml), 1 mM cysteine, 2 mM KCl and 45 mM potassium phosphate buffer (pH 7.4) with or without 0.0067 mM BLM-Fe(II), 0.067 mM BLM-Fe(III) or 0.065 mM BLM-Cu(II) were incubated for 30 minutes at 37°C. The reaction was terminated and the radioactivity of acid-soluble DNA was counted as described in Materials and Methods. Acid-soluble DNA (%) shown in the table is a mean value of duplicate determinations.

Table 3. Effect of BLM-Cu(II) on the generation of malondialdehyde-like product from DNA by BLM-Fe(II) or BLM-Fe(III).

Agents	A_{532}
0.1 mM BLM-Fe(II)	0.281
0.1 mM BLM-Fe(II)+1 mM cysteine	0.438
0.1 mM BLM-Fe(II)+1 mM cysteine+0.025 mM BLM-Cu(II)	0.559
0.1 mM BLM-Fe(II)+1 mM cysteine+0.025 mM $CuSO_4$	0.206
0.1 mM BLM-Fe(III)	0.012
0.1 mM BLM-Fe(III)+1 mM cysteine	0.061
0.1 mM BLM-Fe(III)+1 mM cysteine+0.025 mM BLM-Cu(II)	0.277
0.1 mM BLM-Fe(III)+1 mM cysteine+0.025 mM $CuSO_4$	0.103

The mixtures (0.25 ml) containing 0.5 mg/ml of calf thymus DNA and 50 mM potassium phosphate buffer (pH 7.2) with or without 1 mM cysteine, 0.1 mM BLM-Fe(II), 0.1 mM BLM-Fe(III), 0.025 mM BLM-Cu(II) or 0.025 mM $CuSO_4$ were incubated at 37°C for 60 minutes. After the incubation, the mixtures were combined with 2 ml of 0.6% 2-thiobarbituric acid solution to determine A_{532} as described in Materials and Methods. A_{532} shown in the table is a mean value of duplicate determinations.

soluble DNA produced by 0.067 mM BLM and 1 mM cysteine was increased about 3 times by addition of 0.065 mM BLM-Cu(II). Superoxide dismutase (800 units/ml) did not suppress this increased DNA chain breakage.

As shown in Table 2, DNA chain breakage was increased markedly when 0.067 mM BLM-Fe(III) was combined with 0.065 mM BLM-Cu(II) in the presence of 1 mM cysteine, while in the case of BLM-Fe(II), the addition of BLM-Cu(II) did not give any effect.

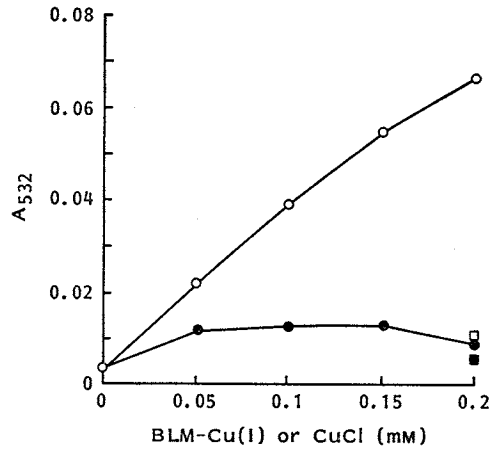
Similar results were obtained in the studies of generation of malondialdehyde-like product from DNA by BLM. As shown in Table 3, 0.1 mM BLM-Fe(II) alone produced the malondialdehyde-like product, and addition of 1 mM cysteine increased the amount of the malondialdehyde-like product about 1.6-fold higher than BLM-Fe(II) alone. Further addition of 0.025 mM BLM-Cu(II) to the mixture increased the amount about 1.3-fold more. When the BLM-Cu(II) was replaced with 0.025 mM $CuSO_4$, the amount of the malondialdehyde-like product was reduced to about one half of that produced by BLM-Fe(II) and cysteine.

BLM-Fe(III) alone gave a trace amount of the malondialdehyde-like product, and BLM-Fe(III) with 1 mM cysteine also produced small amount of the product. Addition of 0.025 mM

Fig. 1. Effect of BLM-Cu(I) or CuCl on the generation of malondialdehyde-like product from DNA by BLM-Fe(III).

The mixtures (0.8 ml) containing 0.5 mg/ml of calf thymus DNA, 0.1 mM BLM-Fe(III) and 50 mM potassium phosphate buffer (pH 7.2) with or without various concentration of BLM-Cu(I) or CuCl were prepared anaerobically under bubbling of N_2 gas. BLM-Cu(I) or CuCl was added last. The mixtures were sealed in test tubes with rubber stoppers against access of air, and incubated at 37°C for 10 minutes followed by aerobic incubation for 10 minutes after mixed with air using a vortex mixer. 0.2 ml aliquots of the mixtures were added to 2 ml of 0.6% 2-thiobarbituric acid solution to determine A_{532} as described in Materials and Methods. Points shown in the figure are mean values of triplicate determinations.

The symbols (□) and (■) in the figure show A_{532} of the incubation mixtures of 0.2 mM BLM-Cu(I) and 0.2 mM CuCl with DNA, respectively. ○ 0.1 mM BLM-Fe(III)+BLM-Cu(I), ● 0.1 mM BLM-Fe(III)+CuCl.



BLM-Cu(II) to the reaction mixture increased the amount of the product about 4.5-folds higher. In the case of addition of 0.025 mM $CuSO_4$ instead of BLM-Cu(II), the increase was only about 1.7-fold of that of BLM-Fe(III) and cysteine.

Effects of BLM-Cu(I) and CuCl on the DNA chain breakage activity of BLM-Fe(III) were next examined in the absence of cysteine. The results are shown in Fig. 1. When BLM-Fe(III) was anaerobically incubated with BLM-Cu(I) followed by aerobic incubation, the malondialdehyde-like product was produced dependently on the concentration of BLM-Cu(I). When CuCl was used instead of BLM-Cu(I), the malondialdehyde-like product was not produced.

Discussion

BLM-Fe(III) is activated by reducing agents[3,4], hydrogen peroxide[4,5] and superoxide anion[5] to cause DNA strand breakage. In the present study, BLM-Fe(III) was shown to be activated by combination of BLM-Cu(II) and cysteine. In the reaction of BLM-Cu(II) with cysteine, BLM-Cu(I) and Cu(I)-complex of cysteine are produced[19,20]. But they are readily oxidized by oxygen yielding superoxide anion[14,20,21]. As presented in this paper, superoxide dismutase gave no effect on the DNA chain breakage increased by combination of BLM with BLM-Cu(II) and cysteine, and combination of BLM-Fe(III) with BLM-Cu(I) in the absence of cysteine caused DNA degradation. Addition of $CuSO_4$ to BLM-Fe(III) and cysteine enhanced DNA degradation, but the degree was much lower compared with the case of the addition of BLM-Cu(II). The effect of $CuSO_4$ seems to be due to formation of BLM-Cu(I) rather than Cu(I)-complex of cysteine because a part of the iron of BLM-Fe(III) is readily displaced by Cu(II)[1] yielding BLM-Cu(I) in the presence of cysteine. Accordingly, among the products in the reaction of BLM-Cu(II) with cysteine, BLM-Cu(I) is thought to activate BLM-Fe(III).

The Fe(III)/Fe(II) redox potential of BLM-iron complex and the Cu(II)/Cu(I) redox potential of BLM-copper complex are +129 mV[22] and −327 mV[23] vs. a normal hydrogen electrode, respectively. Therefore, BLM-Cu(I) is thought to reduce inactive BLM-Fe(III) to active BLM-Fe(II).

Metal-free BLM was reported to become BLM-Cu(II) in the blood after injected into rats[24]. The BLM-Cu(II) incorporated by tumor cells is thought to be transformed to metal-free BLM by reductive liberation of Cu(I) and trapping the Cu(I) with thiol-proteins[18]. A part of BLM-Cu(I) which appears in the process of metal-free BLM formation[19], is expected to reactivate BLM-Fe(III) formed from BLM-Fe(II) after the reaction with DNA as shown in the result that combination of BLM-Cu(II) with BLM-Fe(II) increased amount of malondialdehyde-like product from DNA in the presence of cysteine.

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