Deoxyribonucleic Acid (DNA) Damage Induced by Bleomycin–Fe(II) *in Vitro*: Formation of 8-Hydroxyguanine Residues in DNA

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Treatment of calf thymus deoxyribonucleic acid (DNA) with bleomycin–Fe(II) at 0 °C for 5 min resulted in the formation of 8-hydroxyguanine (8-OH-Gua) residues in DNA in a dose dependent manner, in addition to the formation of base propenal, a DNA-degradation product. The amount of 8-OH-Gua was about one-hundredth of that of base propenal. Treatment of cellular DNA with bleomycin did not result in any increase of 8-OH-Gua even under conditions of 100% cell killing.

Keywords bleomycin; DNA; 8-hydroxyguanine; base propenal; malondialdehyde; active oxygen scavenger; linolenic acid; Ehrlich ascites cell

The bleomycins (BLMs) are a family of glycopeptide-derived antibiotics that have been used clinically against squamous cell carcinomas and malignant lymphomas.[1] The antitumor activity of BLMs is believed to be related to their ability to degrade deoxyribonucleic acid (DNA) by a process whose mechanism has been proposed on the basis of *in vitro* experiments using BLM–Fe(II) and oxygen.[2,3] It has been reported that reactive oxygen species such as $OH^{\cdot}$ and $O_2^{\cdot-}$ are involved in the reaction with DNA.[4] On the other hand, exposure of DNA or cellular DNA to other oxygen-radical generating agents or X-rays is known to result in the formation of several kinds of hydroxylated bases in DNA.[5,6] Among them, the formation of 8-hydroxyguanine (8-OH-Gua),[7] which can be readily measured at high sensitivity[8] and was suggested to be the DNA damage responsible for the induction of mutation,[9] has been extensively studied in several laboratories.[8,10-14] In this paper we report the formation of 8-OH-Gua in DNA treated with BLM–Fe(II) *in vitro*, accompanied with degradation of the sugar moiety. This is the first report to describe the DNA base-damage induced by bleomycin.

Materials and Methods

Chemicals Bleomycin hydrochloride (which contained mainly bleomycin A_2) was purchased from Nippon Kayaku Co., Ltd., Tokyo. Superoxide dismutase (SOD) and catalase were from Sigma Chemical Co., Ltd., St. Louis, Mo. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was used as a source of Fe(II).

Treatment of DNA with BLM–Fe(II) and Measurements of 8-OH-Gua and Base Propenal The reaction mixture (3 ml), containing calf thymus DNA (1 mM·P or 6.5 A_{260}/ml), BLM (0—0.5 mM) and Fe(II) (1—1.2 eq relative to BLM) in 15 mM phosphate buffer (PB) (pH 7.0), was incubated at 0 or 20 °C for 5 or 15 min. Then the mixture was subjected to quantitative analysis for 8-OH-Gua and base propenal. For the measurement of 8-OH-Gua, 0.22 ml of cold 50% trichloroacetic acid (TCA) was added to 2 ml of the reaction mixture. The precipitated DNA was collected by centrifugation (10000 × *g*, 10 min), washed with EtOH and dried. The DNA obtained was enzymatically digested to the component nucleosides, and 8-OH-deoxyguanosine was quantified by high-performance liquid chromatography with an electrochemical detector.[8,14] For the measurement of DNA-degradation products (base propenal or malondialdehyde-like product), 0.5 ml of 15% TCA and 0.5 ml of 100 mM 2-thiobarbituric acid (TBA) sodium salt were added to 1 ml of the reaction mixture and the solution was heated at 95 °C for 15 min. After the solution had been cooled in ice and mixed with 2 ml of H_2O, the absorption of the solution (TBA-condensed product) at 532 nm was measured.[15] Qualitative analysis was performed with the aid of a working curve obtained for the condensate of TBA and malondialdehyde bis(dimethylacetal).

Effect of Active Oxygen Scavengers Treatment of DNA with BLM–Fe(II) or Fe(II) was carried out in the presence of the indicated concentration of a scavenger (SOD, catalase, or mannitol).

Treatment of Linolenic Acid with BLM–Fe(II) The reaction mixture, containing linolenic acid sodium salt (2 mM), BLM (0.1 mM) and Fe(II) (0.1 mM) in 1 ml of PB (pH 7.0), was incubated at 0 or 20 °C for 15 min. Then 0.5 ml of 15% TCA and 0.5 ml of 100 mM TBA were added and the mixture was heated at 95 °C for 15 min. After cooling in ice and addition of 2 ml of 2% sodium dodecyl sulfate solution, the absorption of the solution at 532 nm was measured as described previously.

Treatment of Ehrlich Ascites Cells with BLM Ehrlich ascites cells inoculated i.p. into ICR mice were collected after 1 week. Cells (0.5 ml of packed cells) in 10 ml of PB–saline (pH 7.0) were treated with 0.35 or 0.70 mM BLM at 37 °C for 1 h. Cell survival was checked by two methods; 1) counting the number of trypan blue-excluding cells and 2) measuring the cell growth in ICR mice at 1 week after the i.p. inoculation of the BLM-treated cells (5×10^6). For 8-OH-Gua measurement, DNA was extracted by the Marmur method and subjected to 8-OH-Gua analysis as described previously.

Results and Discussion

When calf thymus DNA was treated with BLM–Fe(II) (molar ratio, 1 : 1.2) in 15 mM phosphate buffer (pH 7.0) at 0 °C for 5 min, 8-OH-Gua residues were formed in proportion to the dose of BLM–Fe(II), until a plateau was reached over the dose of 0.2 mM BLM as shown in Fig. 1. Treatment of DNA with BLM alone did not yield any appreciable amount of 8-OH-Gua, but with Fe(II) alone a

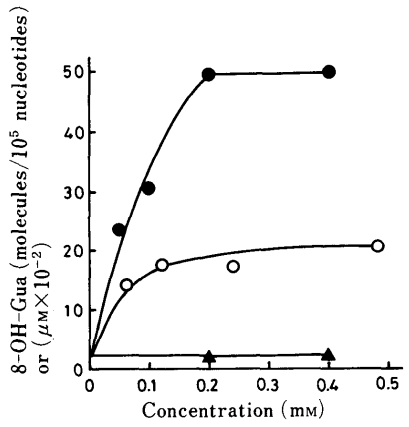

Fig. 1. Formation of 8-OH-Gua in DNA Treated with BLM–Fe(II) (●—●), BLM (▲—▲), or Fe(II) (○—○)

In the case of BLM–Fe(II), the concentration of Fe(II) used was 1.2 eq relative to BLM and the data points are shown at the concentration of BLM. The amount of 8-OH-Gua was expressed as (molecules/10^5 nucleotides) or ($\mu M \times 10^{-2}$ in the reaction mixture containing 1 mM·P DNA).

significant amount was formed, corresponding to about 40% of that with the BLM–Fe(II) mixture. Deducting the amount of 8-OH-Gua produced with Fe(II) alone, the 8-OH-Gua formation due to the presence of BLM at 0.4 mM dosage was calculated to be about 30 molecules/10^5 nucleotides (or 0.3 μM from 1 mM·P DNA), representing a 0.03% yield calculated from starting DNA.

DNA damage measured by the TBA method is shown in Fig. 2. The dose–response curve was almost linear and the amount of TBA-condensed product (a measure of DNA damage leading to strand breakage) reached 28 μM when 0.4 mM BLM–Fe(II) was used. BLM alone did not form any TBA-condensed product, while Fe(II) alone did. At the dose of 0.48 mM, Fe(II) by itself produced 0.6 μM TBA-condensed product, which corresponded to 2% of that produced with 0.4 mM BLM–Fe(II). This is in contrast to the fact that 8-OH-Gua formation by Fe(II) was 40% of that produced by BLM–Fe(II) mixture. Different mechanisms may therefore be involved in the oxidations leading to the formation of 8-OH-Gua and TBA-condensed product, respectively. It is worth noting that there was a great difference between the yields of 8-OH-Gua and DNA degradation. Thus, at 0.4 mM BLM–Fe(II), the product ratio of 8-OH-Gua *versus* TBA-condensed product was 1:91.

The effects of active oxygen scavengers on the formation of 8-OH-Gua and TBA-condensed product were examined at 0 and 20 °C. The results are shown in Tables I and II, respectively. In both cases, the DNA damage induced by BLM–Fe(II) was not appreciably affected by any of the scavengers; SOD for superoxide, catalase for H_2O_2, and mannitol for OH radical. In contrast, the damage induced by Fe(II) alone was definitely reduced in the presence of mannitol, except for 8-OH-Gua formation at 0 °C, indicating that the reactive species involved is the OH radical, as already suggested.[16] These results may allow the conclusion that the reactive oxygen species involved in the bioactivity of BLM is generated by a BLM–Fe(II)–O_2 complex stacked into DNA matrices so as not to allow the scavengers to capture the reactive species generated therein. In other words, the stacking of BLM into DNA matrices may be an obligatory step for DNA damage such as 8-OH-Gua formation and the strand breakage initiated by abstraction of the 4′-hydrogen of the deoxyribose moiety.[17]

Reaction of BLM–Fe(II) with linolenic acid, a membrane

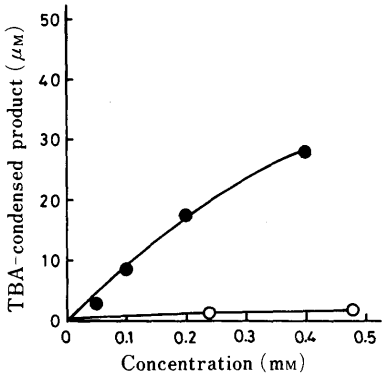

Fig. 2. Formation of DNA Damage Measured by the TBA Method

The amount of TBA-condensed product was expressed as (μM in the reaction mixture containing 1 mM·P DNA). The background value was subtracted. ●—●, BLM–Fe(II); ○—○, Fe(II).

TABLE I. Effect of Active Oxygen Scavengers on the Formation of 8-OH-Gua by Treatment of DNA with BLM–Fe(II) or Fe(II)

	0 °C/5 min		20 °C/5 min	
	8-OH-Gua[a]	%	8-OH-Gua[a]	%
BLM (0.1 mM)+Fe(II) (0.1 mM)	18	100	25	100
+SOD (300 units/ml)	15	83	28	112
+SOD (10 units/ml)	20	111	24	96
+catalase (100 units/ml)	14	78	21	84
+catalase (10 units/ml)	16	89	20	80
+mannitol (10 mM)	17	94	20	80
Fe(II) (0.1 mM)	10	100	16	100
+SOD (10 units/ml)	10	100	13	81
+catalase (10 units/ml)	12	120	—	—
+mannitol (10 mM)	9.5	95	8.3	52

a) Molecules/10^5 nucleotides. The background value (average 4.5) was subtracted.

TABLE II. Effect of Active Oxygen Scavengers on the Formation of DNA Damage Measured by the TBA Method

	0 °C/15 min		20 °C/15 min	
	TBA-condensate[a]	%	TBA-condensate[a]	%
BLM (0.1 mM)+Fe(II) (0.1 mM)	14.9	100	22.1	100
+SOD (10 units/ml)	15.1	101	22.6	102
+catalase (10 units/ml)	15.2	102	22.3	101
+mannitol (10 mM)	14.8	99	21.8	99
BLM (0.1 mM)+Fe(II) (1.0 mM)	30.9	100	31.8	100
+SOD (10 units/ml)	31.7	103	31.6	99
+catalase (10 units/ml)	31.6	102	31.4	99
+mannitol (10 mM)	30.6	99	31.3	98
Fe(II) (1.0 mM)	0.73	100	1.13	100
+SOD (10 units/ml)	0.80	110	1.10	97
+catalase (10 units/ml)	0.70	96	1.15	102
+mannitol (10 mM)	0.27	37	0.35	31

a) μM in the reaction mixture containing 1 mM·P DNA. The background value was subtracted.

TABLE III. Formation of TBA-Condensed Product from Linolenic Acid Treated with BLM–Fe(II)

	0 °C/15 min	20 °C/15 min (μM)[a]
BLM (0.1 mM)	0	0
Fe(II) (0.1 mM)	—	2.2
BLM (0.1 mM)+Fe(II) (0.1 mM)	7.9	9.4

a) Concentration of malondialdehyde-like product produced from 2 mM linolenic acid. The background value was subtracted.

TABLE IV. Treatment of Ehrlich Ascites Cells with BLM. Cell Survival and 8-OH-Gua Formation

BLM (mM)	Survival (%)		8-OH-Gua (molecules/10^5 Nt)
	(A)[a]	(B)[b]	
0	100	100	0.8
0.35	—	—	1.2
0.70	30	0	0.8

a) Determined by counting the number of trypan blue-excluding cells. *b*) Determined by measuring the cell growth in ICR mice as described in Materials and Methods. Nt, nucleotide.

constituent lipid, was examined so as to get basic data relevant to the treatment of cells with BLM. The results are shown in Table III. BLM–Fe(II) was also effective in oxidizing linolenic acid to produce a TBA-condensed product in the present reaction system, although the amount was less than that produced from DNA.

The formation of 8-OH-Gua in cellular DNA was examined using Ehrlich ascites cells in culture. As shown in Table IV, no appreciable amount of 8-OH-Gua was detected; 8-OH-Gua remained at the background level even under conditions of 100% cell killing. Further, under such conditions, the formation of TBA-condensed product was not observed either (data are not shown). It is not clear at present why 8-OH-Gua and TBA-condensed product were not formed in cellular DNA as much as in isolated DNA, and whether or not 8-OH-Gua formation might play a role in the mutagenicity of BLM.[18] One possible explanation for the lack of 8-OH-Gua increase in cellular DNA may be its rapid repair.[14] Another possibility is that the reactive species produced by the BLM–Fe(II) system might be trapped by membrane constituents before they reached DNA (see the *in vitro* experiment with linolenic acid shown in Table III). Most of the DNA damage (except for strand breaks measured by alkaline elution or the centrifugation method) by BLM so far reported has been investigated with isolated but not cellular DNA. It seems that the relationship of BLM–DNA interactions in cells to those studied in *in vitro* systems should be reconsidered.

It has been reported that BLM-mediated DNA degradation is sequence-selective and results in preferential cleavage at –GT– and –GC– sequence sites.[19] We are interested to determine if 8-OH-Gua formation as well as DNA-degradation is sequence-selective.

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