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In Vitro Activation of Bleomycin by Ultraviolet Irradiation

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Irradiation of bleomycin with ultraviolet (UV) light alters its UV absorption and fluorescence spectra and its interaction with deoxyribonucleic acid (DNA) *in vitro*. UV-Irradiated bleomycin decreases the melting temperature (T_m) of DNA and enhances the strand breakage of DNA more markedly than non-irradiated bleomycin, in the presence of sulfhydryl compounds. Moreover, the irradiation dose causing the greatest increase of these activities is in agreement with that causing the greatest quenching at 350 nm in the transition of the fluorescence emission maximum to 400 nm.

Keywords——bleomycin; UV irradiation; UV absorption; fluorescence; DNA melting temperature; DNA strand breakage

Introduction

The bleomycins are glycopeptidic antitumor antibiotics isolated from *Streptomyces verticillus*.[1] Their antitumor activity is considered to be brought about by strand breakage of chromosomal deoxyribonucleic acid (DNA).[2] *In vitro*, bleomycin can bind to DNA, decrease the melting temperature (T_m) of DNA,[3] release free bases and aldehyde from DNA[4–8] and cause strand breakage of DNA in the presence of a reducing agent or ferrous ions.[9–11] Although the mechanisms of interaction with DNA remain unclear, it seems that bleomycin may have bifunctional properties. It is likely that when the bithiazole rings intercalate into double-helical DNA,[12–14] radical(s) such as superoxide or the hydroxyl radical produced from the complex between bleomycin and ferrous ions cause the liberation of free bases and DNA strand breakage.[15–18]

Furthermore, there have been various reports concerning the effects of irradiation of bleomycin with ultraviolet (UV) light.[19–21] These reports have demonstrated that irradiated bleomycin exhibits a decrease of absorbance at 293 nm and a shift of fluorescence emission from 355 to 400 nm, and that DNA strand breakage and the liberation of free bases are enhanced by irradiation when bleomycin and DNA are present together. In this study, the interaction with DNA of bleomycin previously irradiated with UV light was investigated by measuring T_m and the strand breakage activity in the presence of sulfhydryl compounds.

Materials and Methods

Materials——Bleomycin was purchased from Nippon Kayaku Co. and salmon sperm DNA and ethidium bromide were obtained from Sigma Co. Sulfhydryl compounds and other reagents were obtained from Wako Pure Chemical Industries Co. So-called reversible DNA was prepared by Geiduschek's method using HNO_2 treatment for crosslinking.[22] HNO_2–DNA of 90% reversibility was used to assay the strand breakage activity of bleomycin.

Preparation and Spectrometric Analyses of Bleomycin——A solution (3 ml) of 48 μM bleomycin in 50 mM Tris–HCl buffer at pH 7.4 in a cuvette was irradiated with UV light from a 15 W mercury lamp at a distance of 23 cm. Under these conditions, the irradiation dose was estimated at 35 erg/mm^2/s by using the chemical densitometer method. UV absorption and fluorescence spectra were measured with a Hitachi EPS-3T spectrophotometer and an MPF-3 fluorescence spectrometer, respectively.

Assay of Thermal Melting Curve of DNA Treated with Bleomycin——The optical density of a reaction mixture

incubated at 37 °C for 2 h, containing 50 μg/ml salmon sperm DNA ($A_{260}=0.6$), 40 μM previously irradiated bleomycin and 1 mM 2-mercaptoethanol in 50 mM Tris–HCl and 1 mM KCl buffer at pH 7.4 was measured at 260 nm to analyze the thermal melting curve in the temperature range from 37 to 95 °C; the temperature was raised one degree every 2 min.

For measurements of viscosity, 100 μg/ml salmon sperm DNA ($A_{260}=1.2$) was added instead of the 50 μg/ml DNA used for measuring the optical density. The reaction mixture (2 ml) was then placed in an Ostwald-type capillary viscometer and the relative viscosity was measured to analyze the thermal melting curve in the temperature range from 37 to 95 °C; the temperature was rasied one degree every 3 min.

Assay of DNA Strand Breakage Produced by Bleomycin——Changes in the strand breakage activity of DNA were examined by using the method reported by Morgan and Pulleyblank.[23] Reversible DNA ($A_{260}=1.0$) was reacted with 40 μM bleomycin in the presence of 1 mM dithiothreitol in 50 mM Tris–HCl and 1 mM KCl buffer of pH 7.4 at 37 °C for 3.5 h. After incubation, the reaction mixture was denatured in boiling water for 3 min and then quenched immediately in ice. Finally, 500 μg/ml ethidium bromide, dissolved in a solution of 20 mM K_2HPO_4 and 200 μM ethylendiaminetetraacetic acid (EDTA) adjusted to pH 11.5, was immediately added to the reaction mixture. The fluorescence intensity of the final solution of pH 11.3 was measured at 560 nm, with excitation at 525 nm.

Results

When bleomycin was irradiated with UV light, it was clear from the difference spectra that

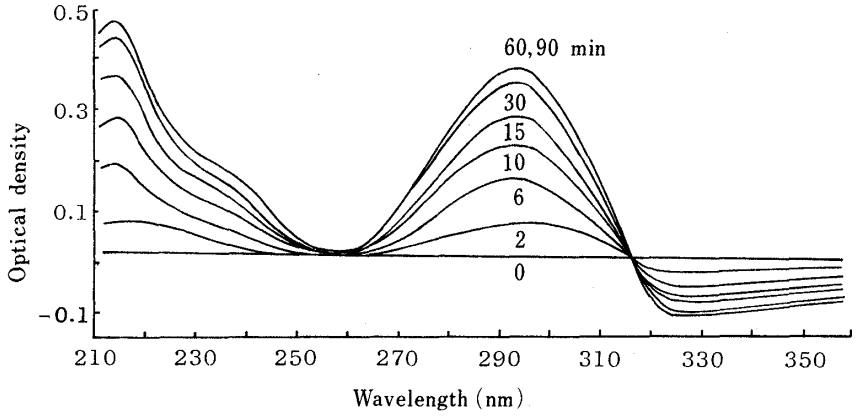

Fig. 1. Effect of UV Irradiation on the Difference UV Absorption Spectra of 48 μM Bleomycin in 50 mM Tris–HCl Buffer at pH 7.4

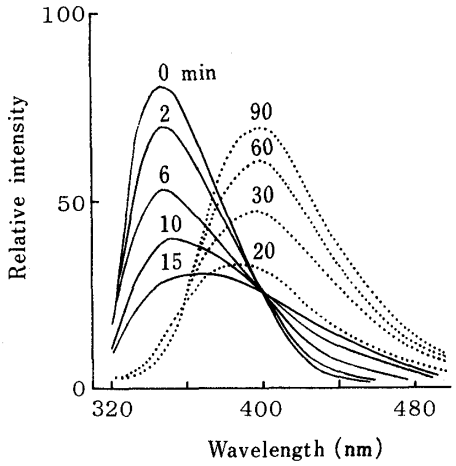

Fig. 2. Effect of UV Irradiation on the Fluorescence of 48 μM Bleomycin in 50 mM Tris–HCl Buffer at pH 7.4

Excitation: 304 nm.

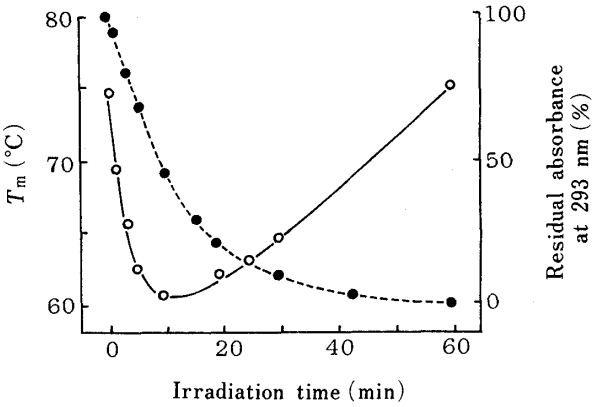

Fig. 3. Effect of 40 μM UV-Irradiated Bleomycin on the T_m of DNA Measured in Terms of Optical Density in the Presence of 1 mM 2-Mercaptoethanol, and the Decrease in UV Absorbance of UV-Irradiated Bleomycin at 293 nm

Left and right scales represent the T_m —○— and the residual absorbance (percent) of UV-irradiated bleomycin at 293 nm ---●---, respectively.

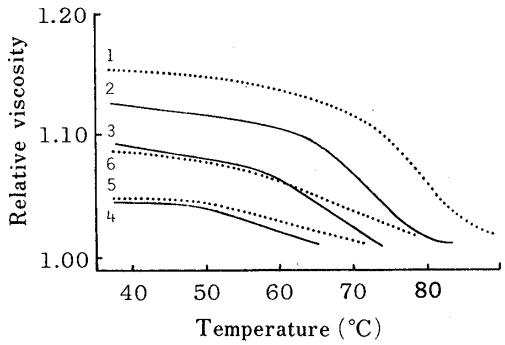

Fig. 4. Thermal Melting Curves of DNA Treated with 40 μM UV-Irradiated Bleomycin in the Presence of 1 mM 2-Mercaptoethanol Measured in Terms of Viscosity

Reaction mixtures: (1) DNA, (2) DNA + non-irradiated bleomycin, (3) DNA + bleomycin irradiated for 1 min, (4) DNA + bleomycin irradiated for 10 min, (5) DNA + bleomycin irradiated for 30 min and (6) DNA + bleomycin irradiated for 60 min.

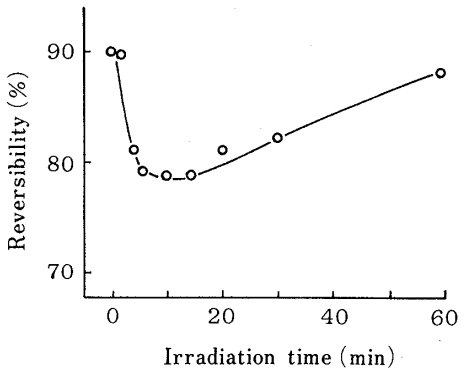

Fig. 5. Plot of DNA Strand Breakage Activity *versus* Irradiation Time for 40 μM UV-Irradiated Bleomycin in the Presence of 1 mM Dithiothreitol

the absorbance at 293 nm decreased markedly as a function of irradiation time, as shown in Fig. 1. Simultaneously, the absorbance at 213 nm, accompanied by a shoulder at 235 nm, also decreased. However, the region at 252 and 262 nm was almost unchanged and an isosbestic point was present at 316 nm.

On the other hand, the fluorescence intensity at 350 nm, excited at 304 nm, was quenched proportionally to irradiation time in the early period. However, as irradiation was further continued, the emission shifted from 350 to 400 nm without any change of the excitation, and the new peak at 400 nm increased as shown in Fig. 2.

In order to study the interaction of irradiated bleomycin with DNA, the thermal melting curves were measured by using the optical density and viscosity methods. It is known that bleomycin can strikingly decrease T_m of DNA in the presence of a reducing agent.[3] As shown in Figs. 3 and 4, the ability of bleomycin to decrease T_m was enhanced by irradiation, and the activity was maximum after about 10 min of irradiation. The result obtained by the viscosity method showed an especially drastic decrease at 37 °C.

It is well known that bleomycin can break DNA strands in the presence or absence of a reducing agent.[9-11] The strand breakage activity of irradiated bleomycin is shown in Fig. 5. The maximum strand breakage activity was produced at the same irradiation dose as that giving the greatest DNA T_m-decreasing ability.

Discussion

Reports have already appeared describing DNA strand breakage and the liberation of free bases by irradiated bleomycin.[20,21] These reports demonstrated an enhancement of the activity by irradiation of bleomycin/DNA. The results obtained in this study show that the activity was enhanced by previous irradiation of bleomycin even in the absence of DNA, and the maximum activity was obtained at a rather short period of irradiation, about 10 min. The viscosity of DNA treated with irradiated bleomycin decreased drastically at 37 °C. DNA strand breakage by previously irradiated bleomycin was also activated, in accordance with the results on T_m-decreasing ability. Interestingly, the maximum activity assesed in terms of both T_m-decreasing ability and strand breakage was produced by the irradiation dose which caused the greatest quenching at 350 nm in the transition of the fluorescence emission maximum to

400 nm. Although it is unclear whether the results of this study relate directly to other investigations on the fluorescence of bleomycin,[24-26] it seems likely that irradiation would cause an alteration of the bithiazole rings. Further, it also seems apparent that activated bleomycin exists as a relatively stable form, and the presence of DNA during the irradiation period is not necessary. Although it has been found that the above effects of bleomycin are activated by a short period of UV irradiation, the inactivation of bleomycin by UV light still remains to be investigated.

References

1) H. Umezawa, K. Maeda, T. Takita and Y. Ohkami, *J. Antibiot. Ser. A*, **19**, 200 (1966).
2) M. T. Kuo, *Cancer Res.*, **41**, 2439 (1981).
3) K. Nagai, H. Suzuki, N. Tanaka and H. Umezawa, *J. Antibiot.*, **22**, 569, 624 (1969).
4) W. E. G. Müller, Z. Yamazaki, H.-J. Breter and R. K. Zhan, *Eur. J. Biochem.*, **31**, 518 (1972).
5) C. W. Haidle, K. K. Weiss and M. T. Kuo, *Molec. Pharmacol.*, **8**, 531 (1972).
6) M. T. Kuo and C. W. Haidle, *Biochim. Biophys. Acta*, **335**, 109 (1973).
7) J. M. C. Gutteridge, *FEBS Lett.*, **105**, 278 (1979).
8) R. M. Burger, A. R. Berkowitz, J. Peisach and S. B. Horwitz, *J. Biol. Chem.*, **255**, 11832 (1980).
9) H. Suzuki, K. Nagai, H. Yamaki, N. Tanaka and H. Umezawa, *J. Antibiot.*, **22**, 446 (1969).
10) C. W. Haidle, *Molec. Pharmacol.*, **7**, 645 (1971).
11) E. A. Sausville, J. Peisach and S. B. Horwitz, *Biochem. Biophys. Res. Commun.*, **73**, 814 (1976).
12) H. Murakami, H. Mori and S. Taira, *J. Theor. Biol.*, **59**, 1 (1976).
13) M. Takeshita, A. P. Grollman, E. Ohtsubo and H. Ohtsubo, *Proc. Natl. Acad. Sci. U.S.A.*, **75**, 5983 (1978).
14) L. F. Povirk, M. Hogan and N. Dattagupta, *Biochemistry*, **18**, 96 (1979).
15) J. W. Lown and S.-K. Sim, *Biochem. Biophys. Res. Commun.*, **77**, 1150 (1977).
16) L. W. Oberley and Buettner, *FEBS Lett.*, **97**, 47 (1979).
17) L. Giloni, M. Takeshita, F. Johnson, C. Iden and A. P. Grollman, *J. Biol. Chem.*, **256**, 8608 (1981).
18) R. M. Burger, J. Peisach and S. B. Horwitz, *J. Biol. Chem.*, **256**, 11636 (1981).
19) N. Thakrar and K. T. Douglas, *Cancer Lett.*, **13**, 265 (1981).
20) K. T. Douglas, N. Thakrar, S. J. Minter, R. W. Davies and C. Scazzochio, *Cancer Lett.*, **16**, 339 (1982).
21) J. Kakinuma, M. Tanabe and H. Orii, *Photobiochem. Photobiophys.*, **7**, 183 (1984).
22) E. P. Geiduschek, *Proc. Natl. Acad. Sci. U.S.A.*, **47**, 950 (1961).
23) A. R. Morgan and D. E. Pulleyblank, *Biochem. Biophys. Res. Commun.*, **61**, 396 (1974).
24) M. Chien, A. P. Grollman and S. B. Horwitz, *Biochemistry*, **16**, 3641 (1977).
25) H. Kasai, H. Nakahara, T. Takita and H. Umezawa, *J. Antibiot.*, **29**, 1316 (1978).
26) C.-H. Huang, L. Galvan and S. T. Crooke, *Biochemistry*, **19**, 1761 (1980).