Free Rad. Res. Comms., Vol. 11, Nos. 4-5, pp. 261-266
Reprints available directly from the publisher
Photocopying permitted by license only

THE ROLE OF REACTIVE OXYGEN SPECIES IN THE ANTITUMOR ACTIVITY OF BLEOMYCIN

H. KAPPUS, D. BOTHE and I. MAHMUTOGLU

Department of Dermatology, Rudolf Virchow Clinic, Free University of Berlin, Augustenburger Platz, D-1000 Berlin 65, F.R.G.

(Received December 15, 1989; in final form June 8, 1990)

Calf thymus DNA was incubated with bleomycin and $FeCl_3$ in the presence of isolated rat liver microsomal NADH-cytochrome b_5 reductase, cytochrome b_5 and NADH which catalyze redox cycling of the bleomycin-Fe-complex. Furthermore, isolated rat liver nuclei were incubated with bleomycin, $FeCl_3$ and NADH, a system in which redox cycling of bleomycin-Fe leads to DNA damage. In both systems free bases from DNA were released. Furthermore, 8-hydroxy-guanine was also found in the supernatant. On the other hand, 8-hydroxy-deoxyguanosine was detected in DNA of cell nuclei indicating that hydroxylation of the guanine molecule occurred in intact DNA. The release of bases correlated with the release of malondialydehyde as well as with NADH and oxygen consumption. These results indicate that NADH-cytochrome b_5 reductase catalyzes redox cycling of the bleomycin-Fe-complex which results in the formation of reactive oxygen species which oxidize deoxyribose as well as bases of DNA. Both mechanisms may contribute to the cytotoxic and cytostatic effects of bleomycin observed in intact cells.

KEY WORDS: Bleomycin, reactive oxygen species, DNA, base release, 8-hydroxy-deoxyguanosine, redox cycling, cell nuclei.

INTRODUCTION

The reduced complex of the anticancer drug bleomycin with iron ions activates oxygen and leads in the presence of DNA to strand breaks induced by reactive oxygen species which oxidize the deoxyribose moiety (for review see [1]). As reaction products base propenals are formed which can be split to free bases and malondialdehyde.[2] In cells cytotoxic and cytostatic effects of bleomycin are presumably due to DNA damage. We have previously shown that two enzymes, NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase are able to catalyze the reduction of the bleomycin-Fe-complex which binds oxygen and leads to oxidation of DNA.[3,4] These enzymes are located in the endoplasmic reticulum as well as in cell nuclei.[5] We suggested that these enzymes are mainly responsible for redox cycling of the bleomycin-Fe-complex resulting in increased oxygen activation and DNA damage. In this respect the most active enzyme was NADH-cytochrome b_5 reductase which was only active in the presence of cytochrome b_5.[4]

We wondered whether during redox cycling by this enzyme free bases are released from DNA indicating oxidative cleavage of DNA by the above mechanism. Furthermore, we were interested whether besides the oxidation of deoxyribose the bases of DNA are oxidized in this system. Therefore, we incubated this enzyme isolated from rat liver microsomes with bleomycin-Fe and DNA. Furthermore, we incubated

Offprint requests to H. Kappus, Department of Dermatology, Rudolf Virchow Clinic, Free University of Berlin, Augustenburger Platz, D-1000 Berlin 65, F.R.G.

isolated rat liver nuclei with bleomycin-Fe and NADH. We measured the release of free bases from DNA in both systems and analyzed and quantified 8-hydroxy-guanine and 8-hydroxy-deoxyguanosine.

MATERIALS AND METHODS

Chemicals and Biochemicals

Bleomycin was the commercially available drug (Mack, Illertissen, F.R.G.). Calf thymus DNA, bases, deoxynucleosides, DNase I (bovine pancreas), endonuclease, phosphodiesterase I, phosphatase (type III S) and TES-buffer were supplied by Sigma, Müchen, F.R.G.. Octyl sulfate was purchased from Aldrich, Steinheim, F.R.G., and DEAE-cellulose from Serva, Heidelberg, F.R.G.. All other chemicals were from Merck, Darmstadt, F.R.G.. HPLC-columns filled with hypersil ODS (3 μm) were obtained from Knaur, Berlin, F.R.G.. 8-Hydroxy-guanine and 8-hydroxy-deoxyguanosine were synthesized according to Kasai *et al.*[6] Cytochrome b_5 and NADH-cytochrome b_5 reductase were isolated from rat liver microsomes as already described.[4] Cell nuclei were isolated from rat liver as described elsewhere.[7]

Incubations

DNA (0.2 mg/ml) was incubated with bleomycin (0.1 mg/ml), $FeCl_3$ (1 mM), $MgCl_2$ (2.5 mM), NADH (1 mM), cytochrome b_5 (0.2 nmoles/ml) and isolated NADH-cytochrome b_5 reductase (0.23 U/ml) in 25 mM TES-buffer (pH 7.5) for 30 min at 37°C.

Rat liver cell nuclei (1 mg nuclear protein/ml) were incubated with bleomycin (1 mg/ml) $FeCl_3$ (1 mM), and $MgCl_2$ (2.5 mM) as above.

Base analysis

At various times samples were withdrawn, centrifuged and treated with DEAE-52-cellulose several times and subjected to HPLC analysis.

DNA was isolated according to a standard procedure including treatment with proteinase K. Deoxynucleosides were split from DNA by adding deoxyribonuclease I and endonuclease followed by treatment with phosphodiesterase I and phosphatase.[8,9] The mixtures of deoxynucleosides were also treated with DEAE-52-cellulose and subjected to HPLC analyses.

Free bases and deoxynucleosides were analyzed by HPLC ion exchange chromatography on hypersil ODS (3 μm) using an eluent of 9% methanol, 3 mM octyl sulfate and 1% H_3PO_4. The bases were detected by UV absorbance at 290 nm, whereas the hydroxylated bases were quantified by electrochemical detection.[10]

RESULTS

Figure 1 demonstrates that during incubation of DNA with the bleomycin-Fe-complex and NADH-cytochrome b_5 reductase all four DNA-bases are released into the medium. This agrees with previous results obtained with NADPH-cytochrome-P-

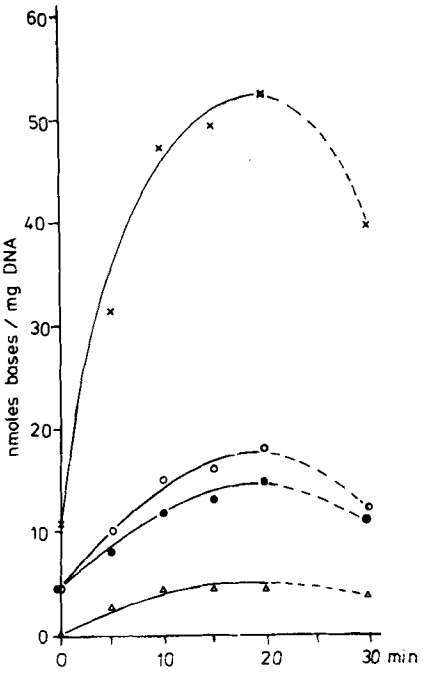

FIGURE 1 Release of free bases from DNA (0.2 mg/ml) incubated with bleomycin (0.1 mg/ml), $FeCl_3$ (1 mM), NADH (1 mM), cytochrome b_5 (0.2 nmoles/ml) and NADH-cytochrome b_5 reductase (0.23 U/ml) thymine (X), guanine (○), adenine (●), cytosine (△)

450 reductase. Parallel to the base release, malondialdehyde was formed and NADH was consumed (data not shown). The release of bases was dependent on NADH, bleomycin-Fe and the enzyme system (data not shown). Table 1 demonstrates that in addition to the release of free bases 8-hydroxy-guanine is also released into the supernatant of the incubation. Per guanine molecule only 1/25 of 8-hydroxy-guanine is measurable (Table 1). It is not known whether hydroxylation of guanine takes place in intact DNA or whether guanine already released from DNA into the supernatant is hydroxylated. This might be clarified by using scavengers of reactive oxygen species

TABLE I

Release of 8-hydroxy-guanine (8-OH-G) from and formation of 8-hydroxy-deoxyguanosine (8-OH-dG) in DNA (0.2 mg/ml) incubated with bleomycin (0.1 mg/ml), $FeCl_3$ (1 mM), NADH (1 mM), cytochrome b_5 (0.2 nmoles/ml) and NADH-cytochrome b_5 reductase (0.23 U/ml).

Incubation time (min)	8-OH-G/nmol G (pmoles)	8-OH-dG/nmol dG (pmoles)
0	0	4
5	11	5
10	22	7
15	38	9
20	—	10
30	46	15

A representative experiment out of 3 is shown.

formed. However, all previous attempts to trap these have not been successful.[3,4,7] On the other hand, Table 1 indicates that hydroxylation of the guanine moiety is possible in the intact DNA and that a small number of molecules of 8-hydroxy-deoxyguanosine is already present in the DNA used.

This amount increases with increasing incubation time. The formation of 8-hydroxy-deoxyguanosine depends on the bleomycin-Fe-complex, the enzyme system and NADH (data not shown). Based on Table 1, we can calculate that over-all only about 1/20 of the guanine molecules present in DNA are hydroxylated.

Figure 2 demonstrates that bases are also released from intact rat liver cell nuclei incubated with NADH. The release of bases into the supernatant was dependent on NADH and the bleomycin-Fe-complex (data not shown). Parallel to the release of bases with incubation time, increasing amounts of malondialdehyde were formed and NADH was consumed as previously[4] described (data not shown). Table 2 demonstrates that also from isolated nuclei 8-hydroxy-guanine is released into the medium when incubated with bleomycin-Fe and NADH. But that hydroxylation of the guanine molecule is not only a secondary reaction step is also shown in Table 2 which demonstrates that hydroxylation of the guanine moiety of DNA occurs also in intact cell nuclei, the hydroxylation step being dependent on the bleomycin-Fe-complex and NADH (data not shown). It can be calculated that about 1/30 of overall guanine molecules in DNA are hydroxylated.

DISCUSSION

From our previous studies it is clear that the bleomycin-Fe-complex undergoes redox cycling in isolated liver cell nuclei.[7] The enzyme responsible for redox cycling is most

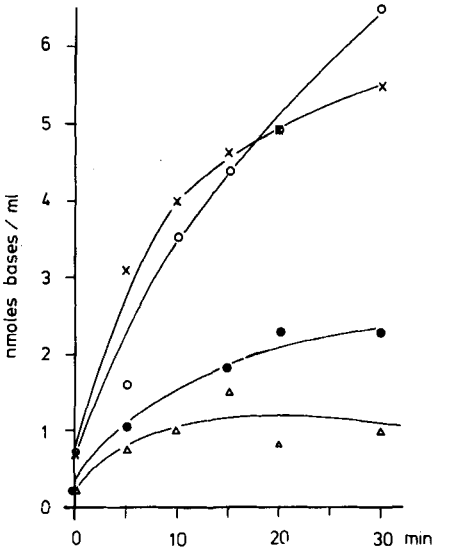

FIGURE 2 Release of free bases from DNA of liver cell nuclei (1 mg nuclear protein/ml) incubated with bleomycin (1 mg/ml), $FeCl_3$ (1 mM) and NADH (1 mM). Base assignment as in Figure 1.

TABLE II

Release of 8-hydroxy-guanine (8-OH-G) from and formation of 8-hydroxy-deoxyguanosine (8-OH-dG) in DNA of liver cell nuclei (1 mg nuclear protein/ml) incubated with bleomycin (1 mg/ml), $FeCl_3$ (1 mM) and NADH (1 mM)

Incubation time (min)	8-OH-G/nmol G (pmoles)	8-OH-dG/nmol dG (pmoles)
0	4	2
5	11	3
10	11	5
15	20	6
20	19	10
30	15	8

A representative experiment out of 3 is shown.

likely NADH-cytochrome b_5 reductase. It has been demonstrated in earlier studies that the deoxyribose moiety of DNA is oxidized by the reduced bleomycin-Fe-complex. This leads to the formation of base propenals which are split to free bases and malondialdehyde.[1,2] Concomitantly strand breaks of DNA occur. Furthermore, the guanine moiety of DNA is hydroxylated.[11] We show here that the release of DNA bases induced by the enzymatically reduced bleomycin-Fe-oxygen-complex is associated with the formation of 8-hydroxy-guanine. It is difficult to quantitate the absolute amounts of hydroxylated bases because we only measured 8-hydroxy-guanine and 8-hydroxy-deoxyguanine. It is likely that also thymine is oxidized leading to the formation of thymine glycol.

However, it is not clear which role oxidation of bases of DNA plays in relation to toxicity. 8-Hydroxy-deoxyguanosine present in intact DNA may result in misrepair and could therefore lead to mutations.[12] On the other hand, it is not very likely that formation of 8-hydroxy-deoxyguanosine induced in cells is responsible for the acute toxicity obsrved with bleomycin.

In summary, during enzymatic redox cycling of bleomycin-Fe a reactive species is formed which is able to oxidize guanine in DNA resulting in the same product, 8-hydroxy-deoxyguanosine, which has been identified after oxidation of DNA by hydroxyl radicals.[13] Therefore, it is likely that the reactive oxygen species formed during redox cycling of the bleomycin-Fe-complex has a similar reactivity as hydroxyl radicals. But it is not known whether this species is really responsible for the cytotoxicity induced by bleomycin in cells.

Acknowledgement

This study has been supported by the Deutsche Forschungsgemeinschaft, Bonn, F.R.G.

References

1. R.M. Burger, J. Peisach and S.B. Horwitz, (1981) Mechanism of Bleomycin Action: In Vitro Studies *Life Science*, **28**, 715–727.
2. A.P. Grollman, M. Takeshita, K.M.R. Pillai and F. Johnson (1985) Origin and Cytotoxic Properties of Base Propenals Derived from DNA, *Cancer Research*, **45**, 1127–1131.
3. M.E. Scheulen, H. Kappus, D. Thyssen and C.G. Schmidt (1981) Redox Cycling of Fe(III)-Bleomycin by NADPH-Cytochrome P-450 Reductase, *Biochemical Pharmacology*, **530**, 3385–3388.
4. I. Mahmutoglu and H. Kappus (1988) Redox Cycling of Bleomycin-Fe(III) and DNA Degradation

by Isolated NADH-Cytochrome b_5 Reductase: Involvement of Cytochrome b_5, *Molecular Pharmacology*, **34**, 578–583.
5. M. Romano, T. Facchinetti and M. Salmona (1983) Is There a Role for Nuclei in the Metabolism of Xenobiotica? A Review, *Drug Metabolism Review*, **14**, 803–829.
6. H. Kasai and S. Nishimura (1984) Hydroxylation of Deoxyguanosine at the C-8 Position by Polyphenols and Aminophenols in the Presence of Hydrogen Peroxide and Ferric Ion, *Nucleic Acids Research*, **12**, 2137–2145.
7. I. Mahmutoglu and H. Kappus (1987) Redox Cycling of Bleomycin-Fe(III) by an NADH-Dependent Enzyme, and DNA Damage in Isolated Rat Liver Nuclei, *Biochemical Pharmacology*, **36**, 3677–3681.
8. F.A. Beland, K.L. Doodley and D.A. Cascano (1979) Rapid Isolation of Carcinogen-bound DNA and RNA by Hydroxyapatite Chromatography, *Journal of Chromatography*, **174**, 177–186.
9. U.J. Nair, R.A. Floyd, J. Nair, V. Bussachini, H. Friesen and H. Bartsch (1987) Formation of Reactive Oxygen Species and of 8-Hydroxydeoxyguanosine in DNA in Vitro with Betal Quid Ingredients, *Chemical Biological Interactions*, **63**, 157–169.
10. R.A. Floyd, J.J. Watson, P.K. Wong, D.H. Altmiller and R.C. Rickard (1986) Hydroxyl Free Radical Adduct of Deoxyguananosine: Sensitive Detection and Mechanisms of Formation, *Free Radical Research Communications*, **1**, 163–172.
11. K. Kohda, H. Kasai, T. Ogawa, T. Suzuki and Y. Kawazoe (1989) Deoxyribonucleic Acid (DNA) Damage Induced by Bleomycin-Fe(II) In Vitro: Formation of 8-Hydroguanine residues in DNA, *Chemical and Pharmaceutical Bulletin, Tokyo*, **37**, 1028–1030
12. Y. Kuchino, F. Mori, H. Kasai, H. Inoue, S. Iwai, K. Miura, E. Ohtsuka and S. Nishimura (1987) Misreading of DNA Templates Containing 8-Hydroxydeoxyguanosine at the Modified Base and at Adjacent Residues, *Nature*, **327**, 77–79.
13. R.A. Floyd, M.S. West, K.L. Eneff, W.E. Hogsett and D.T. Tingey, (1988) Hydroxyl Free Radical Mediated Formation of 8-Hydroxyguanine in Isolated DNA, *Archives of Biochemistry and Biophysics*, **262**, 266–272.

Accepted by Prof. H. Sies