THE PRODUCTION OF HYDROXYL RADICAL BY BLEOMYCIN AND IRON (II)

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1. Introduction

Bleomycin is a glycopeptidic antibiotic used for the treatment of cancer [1]. In vitro, a major effect of bleomycin is the introduction of strand breaks into DNA [2,3]. Efficient breakage of DNA by bleomycin requires the presence of reducing agents, such as 2-mercaptoethanol, dithiothreitol, ascorbate, or hydrogen peroxide [3,4]. Although reducing agents alone are injurious to DNA [5,6], the combination of bleomycin and a reducing agent is far more effective in degrading DNA than is either bleomycin or reducing agent alone [7,8] Limited damage to DNA may be observed with high concentrations of bleomycin alone [8,9].

Since bleomycin binds to DNA in the absence of added reducing agents [10], the role of the latter compounds has recently been studied in detail. It has been established that Fe(II) can substitute for the reducing agents to effectively degrade DNA [11]. Bleomycin and Fe(II) together were far more efficient in cleaving DNA than either species alone. This result has been verified [12]. It was suggested [12], but no convincing evidence was provided, that hydroxyl radical may be responsible for bleomycin-induced damage to DNA.

Fe(III) cannot replace Fe(II) in the degradation of DNA by bleomycin in the absence of reducing agent, but in the presence of reducing agent, either Fe(II) or FE(III) greatly stimulates DNA degradation by bleomycin [11]. Metal chelators and other metal ions inhibit DNA degradation [13]. Superoxide radical (O_2^-) stimulates DNA degradation by bleomycin [13,14]. These observations have led to the proposal of the following model for the action of bleomycin [13]. Bleomycin can bind to DNA in the absence of metal ion or reducing agent. Fe(II) can then attach to the bleomycin and thus form a ternary complex. This ternary complex can then form the species which degrades DNA. Reducing agents enhance the reaction by regenerating Fe(II) from Fe(III) and thus continuing the reaction. Superoxide radical also generates Fe(II) from Fe(III) [15,16]. However, this model does not identify the nature of the toxic species.

Since this proposed mechanism was similar to that observed [16–18] for the production of hydroxyl radical ($^{\cdot}OH$) from xanthine–xanthine oxidase, we thought this radical might also be responsible for the degradation of DNA by bleomycin. We report here the use of the technique of spin trapping [19] to determine the identity of the species produced by bleomycin. The spin trapping technique involves the addition reaction of a short-lived free radical to a compound, spin trap, to produce a relatively long-lived free radical product, spin adduct, which is easily studied by electron spin resonance (ESR). The hyperfine splitting observed enables one in many cases to make a positive identification of the species trapped. Using this technique, we have observed that bleomycin and Fe(II) produce the $^{\cdot}OH$ spin adduct.

2. Materials and methods

2.1. *Reagents*

Bleomycin was obtained from Bristol Laboratories and dissolved in distilled water immediately prior to use. $FeSO_4$ and $FeCl_3$ were obtained from Fisher Scientific. The spin trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) was purchased from Aldrich Chemical

Co. The colored impurity was removed by filtration with decolorizing charcoal using ~10 parts water to 1 part DMPO. The purified DMPO solution was frozen until used.

2.2. *Methods*

All additions were made to 50 mM potassium phosphate buffer (pH 7.8). All reaction mixtures contained ~100 mM DMPO. Solutions in an aqueous sample cell were placed in an electron spin resonance spectrometer (Varian, E-4) cavity, and their spectra obtained using standard procedures.

3. Results

DMPO, 100 mM in potassium phosphate buffer, produced no signal. When either 160 μM bleomycin (250 μg/ml) or 130 μM $FeSO_4$(Fe(II)) was added to the buffer plus 100 mM DMPO, no signal was observed. When 160 μM bleomycin, 100 mM DMPO, and 100 μM Fe(II) was added to the buffer, the signal shown in fig.1 appeared. The spectrum is essentially identical to that identified [20] as the hydroxyl spin adduct of DMPO. When the same experiment is carried out with Fe(III) as $FeCl_3$, no signal results. EDTA, 33 μM, abolished the signal, while 90 μM H_2O_2 enhanced the signal by a factor of ~10. The latter observations are consistent with those in [13].

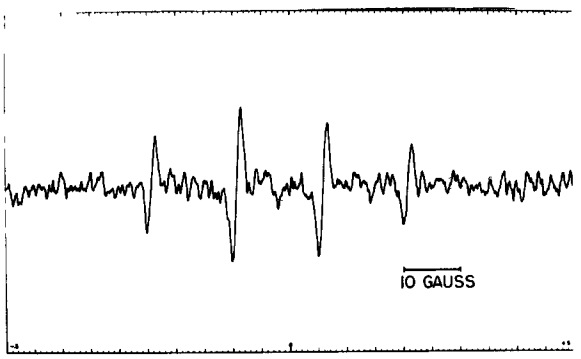

Fig.1. ESR spectra of the hydroxyl spin adduct produced by bleomycin and Fe(II) using DMPO as the spin trap. The reaction mixture contained 160 μM bleomycin, 100 mM DMPO, and 100 μM $FeSO_4$ in 50 mM potassium phosphate buffer (pH 7.8). The spectral properties are identical to those found in [20] for the hydroxyl spin adduct.

4. Discussion

Our results agree with those in [13] and support their model. Fe(II) apparently binds to bleomycin and produces an oxygen-dependent radical. We have identified this radical as ·OH, the most powerful oxidizing radical known to arise in biological systems. Reducing agents, such as H_2O_2, either produce ·OH directly from the reaction between H_2O_2 and Fe(II) (Fenton's reagent), or convert Fe(III) to Fe(II).

This result is analogous to our findings in the xanthine–xanthine oxidase system [16]. In this system, iron or an iron–EDTA complex reacts with superoxide.

$$Fe(III) + O_2^- \rightarrow Fe(II) + O_2$$

$$H_2O_2 + Fe(II) \rightarrow Fe(III) + OH^- + {\cdot}OH$$

Bleomycin evidently reacts with Fe(II) in a similar manner, although EDTA can block this reaction and not the reaction of Fe(II) and H_2O_2.

Since bleomycin binds preferentially to DNA, the net result is that we have a 'site-specific free radical' as proposed [21] for the antitumor action of quinone antibiotics. This release of free radicals in close proximity to DNA is necessary because of the high reactivity of ·OH. Its rate constants are so large for most compounds that it will react with the first molecule it encounters. Thus, in order to damage DNA, it must be released close to it.

As noted earlier, reducing agents such as H_2O_2 or O_2^- are necessary for bleomycin to effectively degrade DNA. The question arises: Where is the source of this reducing agent in the tumor cell? We have recently reported greatly diminished amounts of manganese superoxide dismutase in tumor cells [22,23]. Others have also seen this loss of manganese superoxide dismutase [24,25]. Cu–Zn superoxide dismutase is also reduced in many tumors [26]. This decrease in enzyme activity would result in greatly increased levels of O_2^- in the mitochondria. It has been shown that O_2^- can easily pass through cell membranes [27]. Thus, superoxide produced in the mitochondria may be able to diffuse to the nucleus. Moreover, O_2^- has recently been shown to be produced in tumor cell nuclei [28]. The increased levels of O_2^- in tumor cells as compared to normal

cells may explain the differential toxicity exhibited between normal and malignant cells upon treatment with bleomycin.

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