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SHORT COMMUNICATIONS

Mobilization of ferritin-iron by the anticancer agent bleomycin

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The bleomycins, a group of closely related cytotoxic glycopeptides, are widely used in the treatment of testis-cancer [1]. DNA extracted from cells cultured in the presence of Blm* exhibits strand breaks [2], and the cytotoxic activity of Blm is believed to be closely related to chromosomal and DNA damage. Fragmentation of DNA *in vitro* during incubation with Blm is observed only under conditions where Fe^{2+} (or Fe^{3+} plus an adequate reductant) is present [3, 4]. The iron dependency of the reaction is specific, and an assay for "free" iron has been developed measuring the extent of DNA oxidation obtained in samples containing Blm plus ascorbic acid [5]. Blm has a high affinity for Fe^{3+} ($K_{ass} \sim 10^{15}$) [6], and reactive Blm–iron complexes with ability to bind to DNA are thought to be involved in the reactions leading to DNA damage [7].

The existence of an intracellular chelatable iron pool is debated [8, 9], and the ability of intracellular iron to participate in complex formation with Blm is unknown. The present work was undertaken to examine whether Blm is able to mobilize iron from the intracellular iron storage protein ferritin.

Materials and methods

Materials. Horse spleen ferritin (type 1), calf thymus DNA (type 1), Na_2-bathophenanthroline disulphonate, and Na_2-ADP were from Sigma (St. Louis, MO). 2-Thiobarbituric acid and $FeCl_3 \cdot 6H_2O$ were from Merck (Darmstadt, F.R.G.). Bleomycin sulphate, a mixture of various Blm congeners, was a gift from Lundbeck Ltd. (Copenhagen, Denmark). The drug was dissolved in water assuming a molecular weight of 1550. Ion-exchanged water was used throughout.

EDTA-treatment of ferritin. To ensure that no iron was loosely associated to the ferritin, the protein was treated with 10 mM EDTA in 105 mM KCl/35 mM Tris–HCl, pH 7.4, at 0° for 1 hr the day before use, followed by chromatography on Sephadex G-25 to remove EDTA and extracted iron [10]. Total iron in ferritin stock solutions was determined after sulphuric acid digestion as described in [11].

Incubation conditions. Incubation of Blm and EDTA treated ferritin was carried out in 150 mM KCl/50 mM Tris–HCl buffer, pH 7.4, at 37° in the dark. In experiments involving $FeCl_3$, freshly prepared $FeCl_3$ (0.1 mM in 10 mM HCl) was mixed with Blm (or ADP) 5 min before addition to the incubation mixtures.

Assay for Blm-dependent oxidation of DNA. Fifty microlitres 10 mM ascorbic acid and 200 μl DNA (1 mg/ml) were added to 600 μl samples removed from the incubation mixtures. Oxidation of the DNA was allowed to proceed for 5 min, and was then stopped by the addition of 850 μl 2 mM EDTA. Thereafter, 1 ml 1 M HCl and 800 μl 50 mM TBA (in 100 mM NaOH) were added, and the samples were heated to 95–100° for 10 min. After cooling, the absorption at 532 nm was read against water.

Bathophenanthroline-assay for released ferritin-iron. To 600 μl samples removed from the incubation mixtures at intervals were added 10 μl 10 mM ascorbic acid and 20 μl 50 mM bathophenanthroline disulphonate. The concentration of iron released from ferritin was calculated from the increase in absorbance at 534 nm obtained after 2 min using $\varepsilon = 22.14 \times 10^3\ M^{-1}\ cm^{-1}$ [12].

Absorbance measurements. Spectrophotometric measurements were performed in disposable 1 cm light path cuvettes in a Shimadzu UV-190 double-beam spectrophotometer.

Results and discussion

In our report [13] on the interaction of adriamycin with ferritin we were able to follow transfer of iron to adriamycin spectrophotometrically, due to the intense light absorption at 600 nm of the adriamycin–Fe^{3+} complex ($\varepsilon = 11.4 \times 10^3\ M^{-1}\ cm^{-1}$) [14]. The weaker absorption characteristics of the Blm–Fe^{3+} complex ($\varepsilon = 1.8 \times 10^3\ M^{-1}\ cm^{-1}$ at 430 nm) [15] makes a direct monitoring of formation of Blm–Fe^{3+} complex difficult in the presence of ferritin with significant absorption below 500 nm, and other quantitative estimates of the iron release from ferritin in the presence of Blm were searched for. In initial experiments we therefore determined Blm-dependent mobilization of ferritin-iron using a modification of the DNA-oxidation assay for "free" iron developed by Gutteridge [5]. When DNA was added together with ascorbic acid to mixtures of Blm and Ferritin which had been preincubated at 37° for a 1-hr period, extensive oxidation of the DNA took place (Table 1). The oxidation was dependent both on Blm and ferritin, and was inhibited by EDTA, strongly suggesting that release of iron from the ferritin took place during incubation with Blm. The concentration of "free" iron after the 1 hr incubation was estimated to 1.8 μM when the assay was calibrated with $FeCl_3$.

Phenanthroline-type iron chelators are very suitable as indicators of released ferritin-iron [13, 16, 17]. We therefore examined whether they can be used also in systems including Blm. We found that 1.6 mM bathophenanthroline disulphonate in the presence of 0.16 mM ascorbic acid in 2 min at 37° was able to detect more than 95% of the iron (added as $FeCl_3$) in incubation mixtures also containing 100 μM Blm; thus, the bathophenanthroline disulphonate is able to compete effectively with Blm for complexation of iron. A linear relation between added Fe^{3+} and iron chelated by bathophenanthroline disulphonate was measured up to 10 μM Fe^{3+}. Figure 1 shows the amount of iron detected by the bathophenanthroline assay during incubation of ferritin with various concentrations of Blm. Bathophenanthroline-detectable iron increased with time and with Blm concentration, and reached a maximum after 6–12 hr. The maximal amount of iron (~7 μM) detected in incubation mixtures containing 30 μM Blm corresponds to about 3% of the total iron in the ferritin. 1.7 μM iron was measured after 1 hr incubation of ferritin with 30 μM Blm, close to the concentration of "free" iron (1.8 μM) detected with the DNA-oxidation assay.

The Blm–iron complexes formed during incubation of Blm with ferritin appeared unstable. This might be caused by loss of iron from the Blm [18] with formation of colloids containing iron inaccessible to chelation by bathophenanthroline.

The release of iron from ferritin measured here during

* Abbreviations used: Blm, bleomycin; TBA, 2-thiobarbituric acid.

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Table 1. Oxidation of DNA measured as formation of TBA-reactive material

Incubation mixture	A_{532} (nm)
1. Blm (30 μM) + ferritin (50 μg/ml)	0.290 ± 0.014
2. Blm (30 μM) + Ferritin (50 μg/ml) + EDTA (1 mM)	0.037 ± 0.001
3. Blm (30 μM)	0.030 ± 0.008
4. Ferritin (50 μg/ml)	0.032 ± 0.005
5. Blm (30 μM) + $FeCl_3$ (1.5 μM)	0.255 ± 0.001
6. Blm (30 μM) + $FeCl_3$ (2.0 μM)	0.302 ± 0.016
7. Blm (30 μM) + $FeCl_3$ (2.5 μM)	0.361 ± 0.007
8. ADP (100 μM) + $FeCl_3$ (2.0 μM)	0.002 ± 0.002

DNA and ascorbic acid were added to the incubation mixtures after 1 hr at 37°. Oxidation of the DNA was measured as described under Materials and Methods. Results are expressed as the mean of 3–6 separate experiments ± SD.

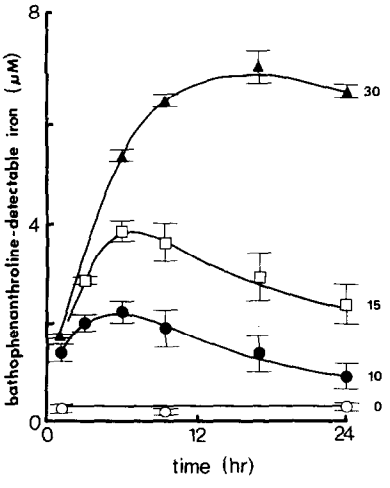

Fig. 1. Mobilization of ferritin-iron by Blm. Ferritin (50 μg/ml) and Blm (0–30 μM as indicated). Released ferritin-iron was measured after the addition of bathophenanthroline as described under Materials and Methods. Values represent the mean of 3–4 separate experiments. Bars are ± SE.

incubation with Blm is comparable to that obtained with adriamycin [13]. Desferrioxamine B, a ferric iron chelator used in the treatment of iron overload, mobilizes ferritin-iron at a similar slow rate [19, 20]. The mechanism by which ferric iron complexing agents can extract iron from ferritin is largely unknown. They may interact with iron at surface sites, but penetration into the ferritin through the pores in its protein shell is also a possibility [21].

In conclusion, the present investigation shows that the cytostatic agent bleomycin is able to interact with an iron pool in isolated ferritin under formation of a DNA damaging bleomycin–iron complex. This finding suggests that ferritin *in vivo* can provide the iron involved in bleomycin induced oxidative DNA damage. It appears therefore important to elucidate further the interaction of bleomycin with ferritin.

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