DNA DAMAGE IN HUMAN COLONIC MUCOSA CELLS INDUCED BY BLEOMYCIN AND THE PROTECTIVE ACTION OF VITAMIN E

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Abstract: Using the alkaline comet assay, we showed that bleomycin at 0.1-5 µg/ml induced DNA strand breaks and/or alkali-labile sites, measurable as the comet tail moment, in human colonic mucosa cells. This DNA damage was completely repaired during a 120-minute post-treatment incubation of the cells. Post-treatment of the bleomycin-damaged DNA with 3-methyladenine-DNA glycosylase II (AlkA), an enzyme recognizing alkylated bases, gave rise to a significant increase in the extent of DNA damage, indicating that the drug could induce alkylative bases in DNA. We did not observe any change in the comet tail moment in the presence of catalase. Vitamin E ((+)-α-tocopherol) decreased DNA damage induced by bleomycin. The results obtained suggest that hydrogen peroxide might not be involved in the formation of DNA lesions induced by bleomycin in the colonic mucosa cells, and that vitamin E may exert protective effects on these cells.

Key Words: Bleomycin, Vitamin E, Human Colonic Mucosa Cells, Catalase, Comet Assay
INTRODUCTION

Bleomycin is a mixture of glycopeptide antibiotics with antitumor activity against testicular cancer, non-Hodgkin's lymphomas, head and neck cancer, Kaposi's sarcoma and cervical cancer [1]. Bleomycin mixtures, which are products of *Streptomyces verticillus*, contain at least 55-75% bleomycin A$_2$ and 25-32% bleomycin B$_2$ (Fig. 1). The clinical effectiveness of bleomycin is based on its cytotoxicity backed up by its interaction with DNA, chelation of metal ions and generation of oxygen free radicals in the presence of molecular oxygen. Bleomycin mainly occupies the minor groove of DNA, with the bithiazole group intercalating between the nucleic bases such that the R group emerges into the major groove. The metal ligands of bleomycin are arranged such that the metal-complexed oxygen is faced to the DNA 4’-hydrogen, which is the unique site of bleomycin attack [2-4]. The bleomycin complex with Fe(II), which can react with oxygen, is probably the most effective complex at DNA degradation. There are at least two modes of bleomycin-induced DNA damage: DNA strand breaks with the excision of base propenal [5], mainly in the G$_2$ phase of cell division, and the generation of alkali-labile products from a C4’-hydroxylated deoxyribose [6, 7]. The first mode requires O$_2$ in addition to the oxygen involved in forming activated bleomycin, while the second does not [8]. The kind of DNA damage induced by bleomycin, such as DNA single- and double-strand breaks, can inhibit DNA replication by both *trans*-(inhibition of replication of the non-damaged template) and *cis*-acting mechanisms (template damage) [9].

Fig. 1. Structure of bleomycin (A) and vitamin E (α-tocopherol) (B).
In this study, we investigated the effect of vitamin E on bleomycin-induced DNA damage in human healthy colonic mucosa cells. Antioxidants such as tocopherols, as effective scavengers of free radicals that are present in colon mucosa, have been suggested to be natural protective agents against DNA damage induced by drugs and compounds originating from the diet. We chose this type of cell because bleomycin can be an effective agent against colon cancer, but it can also induce DNA damage in normal colon cells. We used single-cell gel electrophoresis (the comet assay) to detect DNA damage. The comet assay is used as a sensitive, rapid and relatively simple technique for the evaluation of DNA damage and repair among a variety of cell types, induced by a variety of physical and chemical agents [10]. To search for the mechanism underlying the genotoxic action of bleomycin in the colonic mucosa cells, we applied two enzymes: catalase, which inactivates hydrogen peroxide and 3-methyladenine-DNA glycosylase II (AlkA), an enzyme recognizing and nicking mainly alkylated bases in DNA. We also measured the level of superoxide anion radical generated during bleomycin treatment. The kinetics of the repair of DNA damage induced by bleomycin in these cells was also measured.

**MATERIALS AND METHODS**

**Chemicals**
Vitamin E ((+)-α-tocopherol) from vegetable oil, cytochrome c, Hanks’ balanced salt solution (HBSS), low melting point (LMP) and normal melting point (NMP) agarose, phosphate buffered saline (PBS), catalase and DAPI (4’, 6-diamidino-2-phenylindole) were purchased from Sigma (St. Louis, MO). The Vitamin E used was product type VI. This impure reagent contained approximately 400 mg/gm RRR-alpha-tocopherol plus 5-50 mg/gm non-alpha-tocopherols, plus the remaining 550-595 mg/gm as soybean oil. 3-methyladenine-DNA glycosylase II (AlkA) was donated by Professor Jarosław T. Kusiński of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. Gradisol L was obtained from Aqua Medica (Łódź, Poland).

**Cell isolation**
Human colonic mucosa (CM) cells were obtained from patients undergoing colonoscopy as described elsewhere [11]. The biopsy samples consisted of heterogeneous cell populations including epithelial cells, plasma cells, macrophages and lymphocytes. The experiments were performed on isolated epithelial cells from colonocytes, which amounted to 75% of the total biopsy material. These samples were incubated for 30 minutes at 37°C in a 1.5 ml digestion mixture containing 5.5 mg proteinase K and 3 mg collagenase in 1.5 ml HBSS. Then, they were diluted and centrifuged for 15 minutes at 1500g. The pellet containing colon mucosa cells was resuspended in ice-cold HBSS to give about 10^6 cells ml^-1, and further processed. The viability of the cells was 87%.
The Local Ethics Committee approved the study, and written consent was obtained from each patient before they were enrolled in the study.

**Cell viability**

Cell viability was determined using the trypan blue exclusion analysis. The cells were incubated with bleomycin at concentrations in the range 0.01-20 µg/ml for 1 h at 37°C, washed, and resuspended in Hanks’ balanced salt solution (HBSS). An equal volume of 0.4% trypan blue reagent was added to each cell suspension and the percentage of viable cells was evaluated under a field microscope. Assays were performed in triplicate.

**Chemical treatment**

Bleomycin was taken from stock (0.5 mM) solution and added to the suspension of cells to give final concentrations in the range 0.1-5 µg/ml. To examine DNA damage, the cells were incubated with the drug for 1 hour at 37°C. Each experiment included a positive control, which was hydrogen peroxide at 20 µM, producing a pronounced DNA damage. Vitamin E ((+)-α-tocopherol) was derived from stock (20 mM) solution in dimethyl sulfoxide (DMSO). This was then diluted to 500 µM in the HBSS medium and added to the samples; the final concentration of DMSO in the samples was 0.25% and at this concentration, the chemical did not influence the processes under study (results not shown).

**Comet assay**

The comet assay was performed under alkaline conditions, essentially according to the procedure of Singh et al. (1988) [12] with modifications [13, 14] as described previously [15]. A freshly prepared suspension of human colonic mucosa cells in 0.75% LMP agarose dissolved in PBS was spread onto fully frosted microscope slides (Superior, Germany) precoated with a 0.5% normal-melting agarose. The cells were then lysed for 1 hour at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After the lysis, the slides were placed in an electrophoresis unit, and the DNA was allowed to unwind for 40 minutes in an electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was conducted at an ambient temperature of 4°C (the temperature of the running buffer did not exceed 12°C) for 30 minutes at an electric field strength of 0.73 V/cm (30 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 µg/ml DAPI and covered with cover slips. To prevent additional DNA damage, all the steps described above were conducted under dimmed light or in the dark.

The comets were observed at 200 × magnification in an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to a COHU 4910 video camera (Cohu, San Diego, CA, USA) equipped with a UV-1 filter block (an excitation filter of 359 nm and a barrier filter of 461 nm) and connected to a Lucia-Comet v. 4.51 personal computer-based image analysis system (Laboratory Imaging,
Praha, Czech Republic). Fifty images were randomly selected from each sample. Two parallel tests with aliquots of the same sample of cells were performed for a total of 100 cells. Each experiment was performed three times, so 300 comets were analyzed for each treatment. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample and was used to display the results of all experiments.

**DNA repair**

To examine DNA repair, control samples and cells post-treatment with bleomycin and were washed and incubated in a fresh, bleomycin-free HBSS medium at 37°C. Aliquots of the suspension were taken immediately and 10, 30, 60 and 120 min later. Placing the samples in an ice bath stopped the repair incubation.

**Catalase treatment**

A suspension of the cells in 0.75% LMP agarose was spread onto microscope slides and lysis was performed as described above. After lysis and washing with TE buffer, 50 µl of bleomycin at concentrations of 0.5 and 2 µg/ml with 250 U/ml catalase was spread onto agarose, covered with a cover slip and incubated for 1 hour at 37°C. The slides were then processed as described.

**AlkA treatment**

After incubation with bleomycin and lysis in 2.5 M NaCl, 0.1 mM Na₂EDTA, 10 mM Tris-HCl, 1% Triton X-100, pH 10.0, for 1 hour at 4°C, the slides were washed three times in an AlkA buffer containing 70 mM HEPES-KOH, 1 mM EDTA, 1 mM β-mercaptoethanol, 5% glycerol, pH 7.6. The slides were then drained and the agarose was covered with 25 µl of either buffer or AlkA at 1 µg/ml in buffer and sealed with a cover glass and incubated for 30 minutes at 37°C [16]. Further steps were as described above.

**Superoxide anion radical production**

The production of the superoxide anion radical (O₂⁻) in the cells treated with bleomycin as well as control samples was measured by means of superoxide dismutase-inhibitable reduction of cytochrome c [17]. Cytochrome c at 160 µM in PBS was added to an equal volume of the cell suspension and incubated for 30 minutes at 37°C with bleomycin at concentrations in the range 0.1-5 µg/ml. Reduction of cytochrome c was measured in a Hewlett-Packard UV-visible Spectroscopy System, model HP 8453 (Agilent Technologies, Waldbronn, Germany) at 550 nm. An extinction coefficient for cytochrome c of 18 700 M⁻¹ cm⁻¹ was used to calculate the concentration of O₂⁻.

**Data analysis**

All the values in this study were expressed as mean ± SEM from three experiments. We did not observe differences between results obtained in three
experiments of each treatment. The data from three experiments were pooled and analyzed using one-way ANOVA. A P value of less than 0.05 was considered statistically significant.

RESULTS

Cell viability
The results of cell viability after incubation with bleomycin in the concentration range 0.01-20 µg/ml are shown in Fig. 2. There was a concentration-dependent decrease in cell viability, and for bleomycin at 10 µg/ml, about a half of the cell population was dead (P < 0.001); at 20 µg/ml only about 30% of the cells were viable (P < 0.001). In the concentration range used in further experiments (0.1-5 µg/ml), the viability of the cells was greater than 80%.

![Graph showing cell viability vs. Bleomycin concentration](image)

Fig. 2. The effect of bleomycin on human colon mucosa cell viability measured by the trypan blue exclusion method. Each point is the mean of three experiments; error bars denote SEM. *P<0.05; ***P<0.001.

DNA damage
Fig. 3 shows the mean comet tail moments for the colonic mucosa cells exposed to bleomycin for 1 hour. The drug at a concentration of 1-5 µg/ml significantly increased the comet tail moment (P < 0.001). Hydrogen peroxide at 20 µM for 10 minutes at 4°C (positive control) induced comets in the colonic mucosa cells with a tail moment of 5.6 ± 0.8 (P < 0.001).

DNA repair
Fig. 4 shows the mean comet tail moments of the colonic mucosa cells exposed to bleomycin immediately after exposure as well as 30, 60 and 120 minutes thereafter. In all cases, the comet tail moments of the control cells were constant,
indicating that the preparation and subsequent processing of the cells did not introduce significant damage to their DNA. The cells exposed to 20 µM hydrogen peroxide (positive control) were able to recover within the repair incubation time of 60 minutes (data not shown). The colonic mucosa cells exposed to bleomycin at all tested concentrations repaired damage of their DNA during a 120-minute post-treatment incubation (P < 0.001).

Fig. 3. The mean comet tail moment of human colonic mucosa cells exposed for 1 hour at 37°C to bleomycin. The number of cells in each treatment was 100. Error bars denote SEM. ***P < 0.001.

Fig. 4. Time course of the repair of DNA damage in human colonic mucosa cells treated with bleomycin at 0.1 (●), 2 (□) and 5 mg/ml (■), compared with untreated control (○). The number of cells scored for each treatment was 100. Error bars denote SEM. The statistical significance in the figure is not indicated for the sake of clarity.
Protective action of vitamin E

The mean comet tail moments of the colonic mucosa cells exposed to bleomycin in the presence or in the absence of vitamin E at 20 µM are displayed in Fig. 5. It can be seen that vitamin E significantly decreased the tail moments of the cells exposed to bleomycin at all the tested concentrations of the drug (P < 0.05 for bleomycin at 0.1; 1 and 5 µg/ml and P < 0.001 for bleomycin at 2 µg/ml). In our conditions, pre-incubation of the colonic mucosa cells with vitamin E at 20 µM (1 hour, 37°C) (positive control) decreased the DNA damage induced by hydrogen peroxide at 20 µM (1 hour, 37°C) from 5.6 ± 0.8 to 1.7 ± 0.2 (P < 0.001).

Superoxide anion radical production

Fig. 6 presents the dependence of the concentration of superoxide anion radical on the concentration of bleomycin. The drug did not significantly (P > 0.05) affect the level of the anion, which was equal to about 1 nmol O$_2^\cdot$.

Catalase treatment

The mean comet tail moments for the human colonic mucosa cells exposed to bleomycin with subsequent treatment with 250 U/ml of catalase are shown in Fig. 7. It can be seen that catalase did not significantly reduce (P > 0.05) the tail moment of the colonic mucosa cells exposed to bleomycin at 0.5 and 2 µg/ml.

AlkA treatment

Fig. 8 presents the mean tail moment of the cells exposed for 1 hour to bleomycin with post-treatment with AlkA as compared with that for the cells not
treated with the enzyme. The DNA from the cells incubated with bleomycin at 2 µg/ml and treated with AlkA displayed greater tail moment than the cells not treated with the enzyme (P < 0.01).

Fig. 6. Superoxide anion radical production in human colon mucosa cells exposed for 1 hour at 37°C to bleomycin. Each point is the mean of three experiments; error bars denote SEM.

Fig. 7. The mean tail moment of human colon mucosa cells exposed for 1 hour at 37°C to bleomycin with (filled bars) or without (empty bars) subsequent treatment with catalase at 250 U/ml. The number of cells in each treatment was 100. Error bars denote SEM.
Fig. 8. The mean tail moment of human colon mucosa cells exposed for 1 hour at 37°C to bleomycin with (filled bars) or without (empty bars) subsequent treatment with 3-methyladenine-DNA glycosylase II (AlkA) at 1 µg/ml. The number of cells in each treatment was 100. Error bars denote SEM. **P<0.01

DISCUSSION

The tissues of the gastrointestinal (GI) tract, like all animal tissues, contain superoxide dismutase, catalase and glutathione peroxidase enzymes [18-21]. These enzymes help to detoxify reactive species reaching the gut surface with the diet. However, the intracellular antioxidant defense within the cells of the GI tract cannot provide complete protection against the external effects of oxidants, such as OH·, H2O2, lipid peroxide, aldehydes, HNO2, and oxides of nitrogen [22]. Therefore, supplementation of chemotherapeutic treatment with antioxidants, like vitamins, could enhance the protection of the normal cells of the GI against the attack of damaging species. Vitamins C and E in general absorbed well from the GI tract [23, 24]. It was shown that considerable amount of tocopherols might remain unabsorbed and reach the colon in patients consuming vitamin E supplements [25].

There are many reports that bleomycin can be an effective agent against colon cancer [26-28]. It probably also acts on normal colon cells causing DNA damage. The genotoxic action of anticancer drugs in normal tissues may lead to the induction of secondary malignancies. The problem of recognizing substances that could decrease the genotoxic effects of the anticancer drugs in non-cancer tissues is very important and requires detailed study. On the other hand, bleomycin can damage DNA and may be considered a model agent inducing DNA single- and double strand breaks as well as alkali-labile sites. The colonic mucosa cells are the target cells of many agents that enter the organism along with food. Because colon cancer is among the most frequently occurring
malignancies in the Western world, it is vital to assess the DNA damage in the colonic mucosa cells.

In this study, we investigated the influence of vitamin E ((+)-α-tocopherol) on DNA damage induced by bleomycin in healthy (cancer-free) colonic mucosa cells. The increase in comet tail moment after exposure of the human colonic mucosa cells to bleomycin indicates that this drug can induce DNA breakage and/or alkali-labile sites (Fig. 3). This DNA damage can be repaired during 120-minute post-treatment incubation (Fig. 4). The DNA-damaging potential of bleomycin results from its potential to generate reactive oxygen species such as superoxide and hydroxyl radicals. In the presence of dioxygen and another electron, from the complex of HO\textsubscript{2}-Fe(III)Blm, one hydroxyl radical arises; it can cleave the DNA backbone. The other pathway of DNA damage by bleomycin can occur under anaerobic conditions, and it effectively reduces the peroxide ligand to two water molecules as a base is released from DNA leaving a modified alkaline labile sugar. It has been shown that DNA breakage leads to inhibition of cell proliferation [29]. In cells, bleomycin requires iron for its activation. In preparations of cell nuclei, bleomycin was unable to cause more than 1% of the DNA double-strand breakages resulting from exposure to Fe(III)Blm [30]. In drugs like bleomycin, a redox-active metal ion becomes associated with the target polymers. Thus, bleomycin can generate an activated species of oxygen in the vicinity of DNA; they are responsible for the DNA-damaging potential of bleomycin.

In this study, we did not detect a superoxide anion radical in the colonic mucosa cells incubated with bleomycin (Fig. 6). In the presence of catalase, we did not observe a decrease in the comet tail moments, which suggests that hydrogen peroxide could not be generated in the reaction of bleomycin with DNA and/or could participate in a DNA-damaging reaction by bleomycin (Fig. 7). Our results regarding the role of hydrogen peroxide in DNA damage generation by bleomycin concur with those of other studies. The influence of catalase on the genotoxic effect of bleomycin has been evaluated in three cell lines, which differ in terms of catalase activity [31]. CRL1307, cells from a Xeroderma pigmentosum patient, and CLV102, normal embryonic cells, had catalase activities respectively 3.5 and 5 times lower then CRL2088, normal skin fibroblasts. Bleomycin in the concentration range from 1 to 25 µg/ml, tested in the comet assay, caused a similar degree of DNA damage in embryonic cells and skin fibroblasts. Therefore, the absence of endo- and exogenous catalase influence on bleomycin genotoxicity suggests that not hydrogen peroxide but other reactive oxygen species, like OH\textsuperscript{•}, are formed in the reaction of the activated drug with molecular oxygen [32].

In order to check that bleomycin introduces alkyl base modifications to DNA, we used the bacterial enzyme AlkA. It has broad substrate specificity including 3-methyladenine, 7-methyladenine, 3-methylguanine, O\textsuperscript{2}-alkylcytosine and O\textsuperscript{2}-alkylthymine as well as hypoxanthine, 5-formyluracil and hydroxymethyluracil [33]. AlkA excises methylated bases leaving behind apurinic/apirimidinic sites,
which are converted into strand-breaks and in this way can be detected by the comet assay. The increase in the comet tail moment after incubation of the colonic mucosa cells with bleomycin and treatment with AlkA indicates that this drug generates alkylated DNA bases (Fig. 8).

We observed the decrease in the tail moment of the comets obtained from the cells incubated with bleomycin in the presence of vitamin E ((+)-α-tocopherol) in comparison with the cells incubated with bleomycin only (Fig. 5). This result suggests that vitamin E could protect the human colonic mucosa cells against DNA damage induced by bleomycin. Vitamin E is a naturally occurring group of tocopherols and tocotrienols that function as antioxidants of the lipid phase (Fig. 1). In the case of carcinogens, the protective aspect of vitamin E includes the quenching of free radicals, cell cycle inhibition, the induction of apoptosis and the elimination of tumor cells by an increase in the level of antitumor actions by the immune system [34]. Further, vitamin E can be associated with pro-oxidant properties, principally the degradation of DNA in the presence of copper (II) ions \textit{in vitro} [35]. Our results indicate that vitamin E can protect the DNA of colonic mucosa cells against oxidative stress generated by bleomycin. Earlier studies suggest that vitamin E limited the toxic effects of bleomycin. The most serious side effect of bleomycin is pulmonary injury. It was suggested that supplementation of rats’ diet with α-tocopherol can provide significant protection against bleomycin-induced lung injury [36]. A high dose of vitamin E considerably reduced the fibrotic effect of bleomycin on lung tissue in mice [37]. Short-term vitamin E supplementation, although it causes increased blood levels of alpha-tocopherol, did not provide protection against bleomycin-induced chromosome damage \textit{in vivo} [38].

To our knowledge, this is the first study showing the response of the human colonic mucosa cells to DNA damage induced by bleomycin and the protective effect of vitamin E on this damage. Bleomycin exerts a cytostatic effect on colon cancer. It probably also acts on normal colonic mucosa cells. In this study, we showed that vitamin E could protect these cells against bleomycin. Future studies should investigate the effects of bleomycin treatment on healthy colon cells compared with mutated colon cells. Further studies are also needed to investigate the role of vitamin E on chemotherapy applied in colon cancer. The effects of vitamins on cancer cells are weakly described in the literature. It is not clear that they will decrease the antitumor action of drugs, particularly since certain vitamin E compounds, namely, the tocotrienols and the vitamin E derivative, vitamin E succinate, possess is the ability to induce cancer cells but not normal cells to undergo apoptosis [34].

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REFERENCES


