Protective Effect of Amifostine against Etoposide-Induced Genotoxicity Evaluated By the Comet Assays

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Abstract

Etoposide is one of the most effective chemotherapeutic agents used for the treatment of number of neoplasia. However, as a topoisomerase inhibitor, during clinical use several side effects may occur. In addition in several in vivo and in vitro studies etoposide has shown a range of genotoxic effects including mutation induction and inhibition of DNA synthesis. Amifostine, an organic thiophosphate prodrug, has been shown to exert important cyto-protective effects in many tissues. The aim of this study was to explore whether amifostine protects against etoposide-induced genotoxicity in HepG2 cell line. HepG2 cells (2.5×10^4 cells/well) were cultured in 24-well plates; a control group and three 'amifostine etoposide groups (pre and co- treatment conditions). Our results show that etoposide induced a noticeable genotoxic effect in HepG2 cells. Amifostine reduced the effects of etoposide significantly in both type of experiment conditions, through reduced the level of DNA damage measured via comet and micronucleus assay. Furthermore, amifostine decreased the intracellular ROS generation induced by etoposide. It increased also the intracellular GSH levels in HepG2 cells. Altogether, our results suggest a protective action of amifostine against etoposide cytotoxicity and genotoxicity via various pathways. The most protective effect was observed with amifostine when it was administrated 24 h before etoposide treatment.

Keywords: Etoposide; Amifostine; Comet; Micronucleus; ROS; GSH

Introduction

Etoposide is an anticancer drug used alone or in combination with other drugs in chemotherapy of testicular cancer, lung cancer, lymphoma, leukemia, neuroblastoma and ovarian cancer [1-5]. Side effects of treatment include, vomiting, neutropenia, myelosuppression and alopecia [6-8]. Former studies reported an acute myeloid leukemia as a secondary cancer subsequent treatment by etoposide. Also, etoposide is involved in secondary malignancies because of its genotoxic potential in normal tissues[9, 10].

Topoisomerases are enzymes that catalyze the transient breakage and rejoinging of either one or two DNA strands, to allow one strand to cross another and prevent indeterminable masses during processes such as DNA replication[11, 12]. As a potent topoisomerase inhibitor, etoposide can cause DNA damage by generating a “lesion” that includes DNA strand breaks and protein covalently bound to DNA[13]. Several in vitro and in vivo reports have shown that etoposide induce apoptosis and senescence in different cell lines. Moreover, treatment with etoposide can result in intracellular glutathione (GSH) depletion and increase reactive oxygen species (ROS) generation [14-16]. Etoposide has shown diverse genotoxic effects including mutation induction and several complaints in DNA synthesis in numerous animal and cell line studies [10, 17]. The genotoxicity of etoposide have been evidenced in chromosome aberration tests, micronucleus assays and comet assay in various studies [10, 18, 19]. Second malignancies detected in patients earlier treated with topoisomerase II interactive agents propose these may be a significant clinical outcome of their ability to induce mutation[20].

Amifostine (AME, WR-2721), is a cytoprotective adjuvant used in cancer chemotherapy and radiotherapy involving DNA-binding chemotherapeutic agents [21]. Amifostine is an organic thiophosphate prodrug which is hydrolyzed in vivo by alkaline phosphatase to the active cytoprotective thiols metabolite, WR-1065[22, 23]. According to the several reports, inside the cell, amifostine’s protective effects appear to be mediated by scavenging free radicals, hydrogen donation, induction of cellular hypoxia, the release of endogenous nonprotein sulfhydryl’s (mainly glutathione) from their bond with cell proteins, the formation of mixed disulphides to protect normal cells [24].

Amifostine has shown remarkable radio and chemo protective effects in vitro and in vivo. It is currently approved for clinical use as a protective agent against renal toxicity induced by cisplatin in patients being treated for ovarian cancer and against xerostomia induced by ionizing radiation in patients with head and neck cancer [25-28].

Single cell gel electrophoresis (comet assay) is widely used in genotoxicity testing and is also becoming an important and sensitive tool for evaluating genotoxic potential of compounds such as mutagens and/or antimutagenic agents and carcinogens in vivo and in vitro. In the comet assay, induced DNA damage is evaluated after single cell gel electrophoresis by measuring the tail moment as the product of percent tail DNA multiplied by the tail length of the comet and the percent head DNA. After alkaline lysis, damaged DNA originating from DNA strand breaks and
fluorescence microscope with an excitation filter of 510‐560 nm. Finally comets were visualized under × 400 magnification using ethidium bromide (20 μg/ml ethidium bromide) for 5 min and washed with distilled water.

The slides were covered by sufficient dye solution (20 μg/ml) for 10 min. The slides were placed in the neutralization solution (pH=7.5) for 10 min. Electrophoresis was conducted for 40 min at 25 V with an electricity current adjusted to 300 mA. After this stage, the slides were washed twice with HBSS and incubated in 2 ml of fresh culture medium without FBS. 2_, 7_. Dichlorodihydrofluorescein diacetate was added at a final concentration of 10μM and incubated for 20 min. The cells were then washed twice with PBS and maintained in 1 ml of culture medium. Assess ROS by immediately analyzing cells by fluorescence plate reader using the 488 nm for excitation and detected at 535 nm. We have chosen untreated cells as a negative control and cells treated with 0.1 mM H2O2 as a positive control.

Measurement of intracellular GSH levels

HepG2 cells were plated in a 96-well plate at 50,000 cells/well. After overnight growth, they were treated with test vehicles and then incubated with monochlorobimane (mBCI, 40 μM) in a staining solution (5mMglucose, 1 mM CaCl2, 0.5mMMgSO4, 5 mg/ml BSA) for 30 min at 37°C in the dark. Although mBCI is a nonfluorescent probe, it forms a stable fluorescent adduct with GSH in a reaction catalyzed by the GSH S-transferases. The mean fluorescent intensity of the fluorescent GSH-bimane adduct was measured using a Spectra fluorescent plate reader at λex=380 nm and λem=460 nm to detect GSH. The assay was performed for amifostine for studied concentration (1, 5 and 10 mg/ml) and etoposide (1µM) in pretreatment condition.

Statistical analysis

Tail moment (percentage of DNA in the tail ×tail length), tail length (the length of the comet tail), and percent of DNA in the tail (percentage of colored spots in tail) are the most frequently used factors in the evaluation of DNA damages in the comet assay method. We used these factors for statistical analysis in this investigation. One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison post hoc tests was used to compare the results of all assays. Value of p < 0.05 was considered to be significant.

Results

Study the effect of amifostine on etoposide-induced DNA damage

The anti-genotoxic effect of amifostine was investigated through the alkaline comet assay. Results of the visual scoring and percentage of total DNA damage induced by etoposide and prevented by amifostine were shown in Table 1. We observed that etoposide treatment at 1μM induced a significant (p < 0.001) increase in DNA damage as compared to the control group. Amifostine in the different treatment conditions decreased...
significantly (p < 0.0001) the level of DNA fragmentation as compared to the etoposide group.

**Study the effect of amifostine on ROS generation in etoposide-treated cells**

To investigate the role of oxidative stress in etoposide-induced genotoxicity, we used DCFH-DA, a cell-permeable fluorescent dye, to examine the ROS generation in HepG2 cells in response to etoposide stimulation. Incubation with etoposide for 1 h showed a considerable increase in oxidant-induced 2-, 7,-dichlorofluorescein fluorescence in HepG2 cells (Figure 1). H2O2-mediated DCF fluorescence occurred after 1 h incubation with etoposide (1µM) in HepG2 cells. This suggests that etoposide, induce intracellular oxidative stress, involved in its genotoxicity. After that cells were treated with amifostine in pre-treatment condition and subsequently examined. Amifostine was significantly (p<0.0001) reduced ROS generation as compared to the etoposide group. Untreated cells served as control.

**Study the effect of etoposide on intracellular levels of GSH**

We first examined the effect of etoposide on the intracellular levels of GSH using mBCI which readily enters cells to form a fluorescent GSH-bimane adduct that can be measured fluorometrically. As shown in fig.2, within 1 h after etoposide (1µM) treatment, the intracellular levels of GSH were reduced (p<0.0001). This finding was subsequently confirmed by an enzymatic assay using glutathione reductase and 2-vinylpyridine. Next; we measured the intracellular levels of GSH in cells after treatment with amifostine and etoposide in pre-treatment condition. As shown in figure 2 amifostine were significantly (p<0.0001) increased GSH levels as compared to the etoposide group.

**Discussion**

Etoposide as a chemotherapeutic agent is used in combination with other anticancer drugs in the treatment of many cancerous diseases such as testicular, bladder, prostate, lung, and uterine cancers [32, 33]. It seems that etoposide act as a topoisomerase II inhibitor, although the clear mechanism of action of this drug is not known [34]. Also, etoposide can induce secondary malignancies due to its genotoxic potential in normal tissues. As a strong topoisomerase II inhibitor agent etoposide can cause DNA damage by different mechanisms. The main mechanism of

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**Table 1: The genoprotective effect of Amifostine compared with control groups on tail length (pixels), percentage of DNA in tail, and tail moment (pixels) that are represented as mean±SEM. The sign (*) show significantly (p<0.0001) decreased compared to the etoposide group. (One-way ANOVA followed by tukeys post hoc test)**

<table>
<thead>
<tr>
<th>Pre-treatment</th>
<th>Treatment</th>
<th>Tail length (Pixels) (Mean±SEM)</th>
<th>%DNA in Tail (Mean±SEM)</th>
<th>Tail moment (Mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Etoposide 1µM)</td>
<td>134.1±1.1</td>
<td>69.5±1.9</td>
<td>41.3±0.8</td>
<td>54.2±1.75</td>
</tr>
<tr>
<td>Amifostine (1mg/ml)</td>
<td>79.11±0.9</td>
<td>14.2±0.3</td>
<td>8.1±0.7</td>
<td>1.19±0.7</td>
</tr>
<tr>
<td>Amifostine (5mg/ml)</td>
<td>32.12±0.9</td>
<td>41.3±0.8</td>
<td>8.1±0.7</td>
<td>1.19±0.7</td>
</tr>
<tr>
<td>Amifostine (10mg/ml)</td>
<td>18.2±1.8</td>
<td>9.9±0.1</td>
<td>1.19±0.7</td>
<td>1.19±0.7</td>
</tr>
</tbody>
</table>

Figure 1: Study the effect of amifostine on etoposide-induced ROS generation. (****) show significantly increased results (respectively p<0.0001) as compared to the control group. The sign (#) show significantly (p<0.0001) decreased compared to the etoposide group.

Figure 2: The effect of amifostine on the levels of intracellular GSH was determined. ANOVA analysis revealed that amifostine, significantly inhibited the effects of amifostine on the levels of GSH. Sign (***) and (*) show significantly decreased results (respectively p<0.0001 and p<0.05) as compared to the control group. Sign # show significantly (p<0.0001) increased as compared to the amifostine group.
action proposed for topoisomerase II inhibitor as etoposide is steady the double-stranded DNA cleavage normally catalyzed by topoisomerase II (topo II) and inhibit accurate relegation of DNA breaks. These double-strand DNA breaks not only consequently prompt the anticipated antitumor effects of the drugs but also interact with normal cells and induce genotoxicity [35,36].

Amifostine, is the most effective radioprotector known and the only one accepted for clinical use in cancer radiotherapy [37]. This antigenotoxic effect was explained by assuming a high affinity of amifostine for DNA, thereby stabilizing the DNA molecule and facilitating the activity of DNA repair enzymes [38]. Previous studies using mammal cells have shown that amifostine enhances DNA repair and thus improves cell survival. Amifostine phosphorylated aminothiol, also is an antioxidant clinically prescribed to prevent the neutropenia-associated events in patients receiving alkylating agents [39]. In experimental animals, Yuhas and Storer showed that treatment with AMF effectively protects normal tissue from the toxicity of therapeutic radiation, without protecting tumor [40]. Nagy et al. subsequently showed that AMF showed the protective effect against the mutagenicity of cisplatin, evaluated by the mutation rate of HPRT in V79 Chinese hamster cells [41]. Other reports documented that amifostine protects normal tissue against radiation-induced damage by increasing intracellular SOD2 activity. Once dephosphorylated by the membrane-bound alkaline phosphatase (ALP), AMF is activated to a free thiol form (WR-1065), which is preferentially taken up by normal cells, since ALP is more active and efficiently expressed in normal rather than neoplastic tissue [42]. Moreover, in another study found that WR1065, the active free thiol form of amifostine, induces antioxidative ability against radiation via SOD2 in vitro [43].

In our investigation we quantified the DNA-damage level, to elucidate the possible anti-genotoxic mechanism of amifostine against etoposide-induced toxicity in HepG2 cell line. Our results showed that etoposide alone caused a significant increase in DNA fragmentation as compared to the untreated cells. However, treatment of HepG2 cells with amifostine 24 h before etoposide administration induced a noticeable decrease in DNA fragmentation as compared to the etoposide-treated group. Measurement of ROS generation showed that etoposide induced ROS generation. Amifostine is a potent cytoprotective agent that can inhibit oxidative stress by scavenging ROS and replenishing GSH.

Conclusion
In conclusion, we have demonstrated that amifostine protected HepG2 cells against etoposide-induced DNA damage and oxidative injury. Furthermore, we showed that etoposide increased intracellular ROS generation and decreased in intracellular GSH levels. Amifostine ameliorated the balance of intracellular antioxidants and oxidants, decreased ROS generation and enhanced the intracellular level of GSH. Altogether, our results suggest a protective action of amifostine against etoposide cytotoxicity and genotoxicity via various pathways. The most protective effect was observed with amifostine when it was administrated 24 h before etoposide treatment.

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