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Research Article

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Antioxidant and Genoprotective Effects of Amifostine against Irinotecan Toxicity in Human Hepatoma Cells

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Abstract

Irinotecan (CPT-11) is a topoisomerase inhibitor anticancer drug effective against many human malignancies. Several mechanisms have been proposed for the antitumor effects of irinotecan, such as DNA synthesis inhibition, DNA crosslinking, inhibition of topoisomerase I, free radical generation and lipid peroxidation. Amifostine, is a cytoprotective adjuvant used in cancer chemotherapy, involving DNA-binding chemotherapeutic agents. The aim of this study was to explore whether amifostine protects against irinotecan-induced genotoxicity in HepG2 cells. For this purpose, we measured the DNA damage level with comet assay in HepG2 cells treated with irinotecan and amifostine in different condition. We also measured the intracellular ROS generation and GSH levels in cells treated with irinotecan and amifostine in pre-treatment condition. Our results showed that irinotecan induced a noticeable genotoxic effect in HepG2 cells. Amifostine reduced the effects of irinotecan significantly ($p<0.0001$) by reduction of the level of DNA damage via blocking ROS generation, and enhancement of intracellular glutathione levels.

Keywords: Irinotecan, Amifostine, Comet assay, ROS, Genotoxicity

Introduction

Irinotecan (CPT-11) is a semisynthetic, water-soluble derivative of camptothecinwith antineoplastic effect [1]. Irinotecan, as a topoisomerase I (TOP1) inhibitor, is used in the treatment of various types of cancers such asmetastatic colorectal and ovarian carcinoma [2]. Side effects of treatment include myelosuppression, neutropenia, nausea, vomiting and induction of secondary tumor [3-5]. Binding of irinotecan to topoisomerase II results in cleavable complexes that generate DNA strand breaks, inhibits DNA replication and RNA transcription in a cell cycle nonspecific manner [6,7]. Irinotecan cause apoptosis, mitochondrial dysfunction, and free radical generation in normal cells as well as tumorous cells [8-10]. The ability to induce DNA damage in normal cells and the induction of secondary malignancies may be considered as the most critical side effects of anticancer drugs [5,11]. The genotoxic effects of irinotecan have been proven by chromosomal aberration tests, micronucleus assay and Comet assay in various studies [11-13]. Thus, a thorough assessment aimed to its side effects, like genotoxicity which leads to secondary malignancy is required.

Amifostine, is a cytoprotective agent used in cancer chemotherapy and radiotherapy involving DNA-binding chemotherapeutic agents [14]. Amifostine is an inactive prodrug that cannot protect cells until dephosphorylated to the active metabolite, WR-1065, by alkaline phosphatase in the plasma [15]. According to the different studies, 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 and formation of mixed disulphides to protect normal cells [16]. Amifostine has shown significant radio- and chemoprotective effects in several in vitro and in vivo studies. It is presently accepted 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 [17-20]. Preclinical studies have shown that administration of amifostine before irradiation protected against radiation clastogenesis, mutagenesis and carcinogenesis [21,22]. Amifostine is able to inactivate electrophilic substances and scavenge free radicals [23]. In addition numerous studies has been showed that amifostine attenuate cardiotoxicity, nephrotoxicity and genotoxicity result from chemotherapy agents [18,24-26].

Single cell gel electrophoresis (comet assay) is considered as some sensitive methods for analyzing genotoxic or genoprotective potential of compounds is normally used in genotoxicity testing. Applications of this test include genotoxicity testing, human biomonitoring and molecular epidemiology, ecogenotoxicology, as well as primary research in DNA damage and repair [27,28]. The purpose of present study was to explore the protective effect amifostine against irinotecan induced genotoxicity. For this purpose, we measured the DNA damage level with comet assay in HepG2 cells treated with irinotecan and amifostine in co and pre-treatment conditions. We also investigated the generation of ROS and intracellular glutathione

levels as possible genotoxic mechanisms.

Materials and Methods

Chemicals

irinotecan was purchased from Sigma-Aldrich, France. Amifostine, EDTA, H2O2, NaCl, NaOH, Na2CO3, NaH2PO4, Tris, and Triton X-100 were acquired from Merck Co. (Germany). Low melting point agarose (LMA), Na2HPO4, KCl and ethidium bromide were from Sigma Co. (USA). Normal melting point agarose (NMA) was supplied by Cinnagen Co (Germany). The RPMI 1640 medium, fetal bovine serum (FBS) and the antibiotic were purchased from biosera (France). DCFH-DA probe and mBCl were from sigma Aldrich (USA) And, HepG2 cells came from Pasture Institute (Iran). All other chemicals used were of analytical grade.

Cell culture

Human hepatoma (HepG2) cells were obtained from Pasture Institute of Iran were grown as monolayer culture in RPMI 1640 medium supplemented with 10% FBS, 1% of mixture of penicillin (100 IU/ml) and streptomycin (100µg/ml) incubated at 37◦C in an atmosphere of 5% CO2–95% air mixture. Amifostine was dissolved in the cell culture medium. We have chosen untreated cells as a control. Cells were seeded in 24-well culture plates at 25×10 4 cells/well, after overnight growth, cells treated with studied concentrations of amifostine (1,5 and 10 mg/ml) 24 h prior and Simultaneously to irinotecan treatment (100µM) for 1 h at 37 ◦C [28].

Single-cell gel electrophoresis (SCGE, the comet assay)

The comet assay procedure has been described in our previous studies [29-32]. Briefly, incubated cell suspensions (1 × 106 cells/ml) were mixed with 1% LMP agarose at 37ºC, were placed on the precoated slides (1% NMP agarose), and covered by cover glasses for 5 min at 2-8 °C. The slides were incubated with lysis solution (pH=10.0) for 40 min and rinsed with distilled water to remove the excess lysis solution. In the next step, slides were incubated with electrophoresis buffer (pH> 13.0) for 40 min. Electrophoresis was conducted for 40 min at 25 V with an electricity current adjusted to 300 mA. After this stage, the slides were rinsed with distilled water to remove excess alkaline buffer and were placed in the neutralization solution (pH=7.5) for 10 min. The slides were covered by sufficient dye solution (20 µg/ml ethidium bromide) for 5 min and washed with distilled water. Finally, comets were visualized under × 400 magnification using fluorescence microscope with an excitation filter of 510-560 nm and the barrier filter of 590 nm [23]. All stages of comet assay were performed in dark conditions and all solutions were prepared freshly and used cool.

Measurement of Oxidative Stress

Approximately 4 × 10^4 cells per well were cultured for 24 h in 96-well plates (black-wall/clear-bottom). Thereafter, the medium was aspirated, and the cells were washed twice with HBSS. The cells were then treated with studied concentrations of amifostine (1, 5 and 10 mg/ml) 24 h prior irinotecan treatment (100µM) for 1 h at 37 ◦C. After the treatment, cells 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 [28].

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 irinotecan (100µM) in pretreatment condition [28].

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 irinotecan-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 irinotecan and prevented by amifostine were shown in Table 1. We observed that irinotecan treatment at 100µM 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 control group.

	Treatment	Tail length (Pixels) (Mean ±SEM)	%DNA in Tail (Mean±SEM)	Tail moment (Mean±SEM)
Pre-treatment	Control (CPT11 100µM)	143.1±2.673	7±2.750.	3±1.76
	Amifostine (1mg/ml)	81.11±2.4	63.2±1.3	46.3±1.3
	Amifostine (5mg/ml)	29.42±1.1 *	14.4±0.6*	12.1±1.6*
	Amifostine (10mg/ml)	18.2±1.33*#	5.1±0.6*	1.2±.037*
Co-treatment	Control (CPT11 100µM)	143.1±2.673	7±2.750.	3±1.76
	Amifostine (1mg/ml)	90.5±1.266	37±1.1	41.45±1.2
	Amifostine (5mg/ml)	31.6±1.6*	19±0.57*	7.2±1.2*
	Amifostine (10mg/ml)	21.43±1.7*	8.3±0.5 *	1.9±.02*

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 irinotecan group. (one-way ANOVA followed by tukeys post hoc test).

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

To investigate the role of oxidative stress in irinotecan -induced genotoxicity, we used DCFH-DA, a cell-permeable fluorescent dye, to examine the ROS generation in HepG2 cells in response to irinotecan stimulation. Incubation with irinotecan for 1 h showed a considerable increase in oxidant-induced 2_, 7_-dichlorofluorescein fluorescence in HepG2 cells (Fig. 1). H2O2-mediated DCF fluorescence occurred after 1h incubation with irinotecan (100µM) in HepG2 cells. This suggests that irinotecan, 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 irinotecan group. Untreated cells served as control.

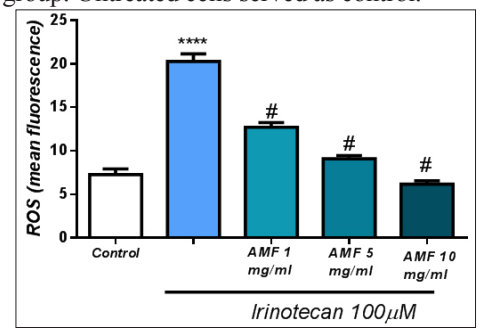

Figure 1: Study the effect of amifostine on irinotecan-induced ROS generation. (****) show significantly increased results (respectively $p<0.0001$) as compared to the control group. The sign (#) show significantly ($p<0.0001$) decreased compared to the irinotecan group.

Study the effect of irinotecan on intracellular levels of GSH

We first examined the effect of irinotecan 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 1h after irinotecan (100µM) 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 irinotecan in pre- treatment condition. As shown in fig.2 amifostine were significantly ($p<0.0001$) increased GSH levels as compared to the irinotecan group.

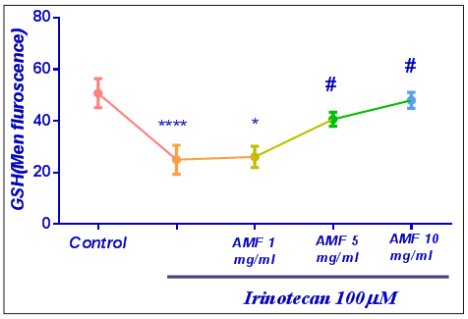

Figure 2:The effect of amifostine on the levels of intracellular GSH were determined. ANOVA analysis revealed that amifostine, significantly inhibited the effects of irinotecan on the levels of GSH. Sign (****) and (*) show significantly decreased results (respectively $p<0.0001$and $p<0.05$) as compared to the control group. Sign # show significantly ($p<0.0001$) increased as compared to the irinotecan group.

Discussion

In cancer treatment, irinotecan is a commonly used drug against several human malignancies such as colorectal and ovarian carcinoma [2]. Genotoxic drugs affect both normal and cancer cells, but the selectivity associated with sensitivity of rapidly dividing cells such as cancer cells [29]. The importance of cancer cell-specific mechanism intended agents such as inhibitors of DNA topoisomerases which are the major class of anticancer drugs is increasing.Topoisomerase inhibitors act by transiently trapping the enzymes in these intermediate complexes, often preventing the nicks from re-ligating and leading to DNA strand breaks [30,31]. Therefore, our study had three general aims. Firstly, we tried to assess the ability of irinotecan to damage DNA in human hepatoma cells. Secondly, we explored the protective effect of amifostine against DNA-damaging effects evoked by irinotecan. Thirdly, we attempted to evaluate the protective potential of amifostine against generation of ROS and depletion of intracellular glutathione levels as the probable genotoxic mechanism. Our experimental data indicate that irinotecan can generate damage to DNA in HepG2 cells ($p<0.0001$). It is likely, that the damage is caused by oxygen radicals generated by irinotecan; DNA methylation by the drug can also contribute to the damage.

Amifostine, is the most effective radio protector known and the only one accepted for clinical use in cancer radiotherapy [32]. This ant genotoxic 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 [33]. 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 [34]. In experimental animals, Yuhas and Storer showed that treatment with AMF effectively protects normal tissue from the toxicity of therapeutic radiation, without protecting tumor [35]. 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 [36]. 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 up taken by normal cells, since ALP is more active and efficiently expressed in normal rather than neoplastic tissue [37]. Moreover, in another study found that WR1065, the active free thiol form of amifostine, induces antioxidative ability against radiation via SOD2 in vitro [38,39]. Other studies have been shown the role of SOD2 in amifostine-induced protective effects, SOD2 mediated amifostine-induced antioxidative actions in PC12 cells exposed to glutamate. As SOD2 protein is mainly expressed in mitochondria which have been identified as a major source of ROS, we infer that high level of SOD2 protein may protect mitochondria by consuming ROS generated in oxidative injury. In addition, SOD2 mediated amifostine-induced effects on intracellular ROS, CAT, and GSH levels, indicating SOD2 may be the key target

of amifostine in maintaining the balance of intracellular oxidants and antioxidants in PC12 cells. In our investigation we quantified the DNA-damage level, to elucidate the possible anti-genotoxic mechanism of amifostine against irinotecan -induced toxicity in HepG2 cell line. Our results showed that irinotecan alone caused a significant increase in DNA fragmentation as compared to the untreated cells. However, treatment of HepG2 cells with amifostine 24 h before irinotecan administration induced a noticeable decrease in DNA fragmentation as compared to the irinotecan -treated group. Measurement of ROS generation showed that irinotecan 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 irinotecan-induced DNA damage and oxidative injury. Furthermore, we showed that irinotecan increased intracellular ROS generation and decreased intracellular GSH levels. Amifostine ameliorated the balance of intracellular antioxidants and oxidants, decreased ROS generation and enhanced the intracellular level of GSH.

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