Iran. J. Radiat. Res., 2005; 3 (1): 31-36

Effect of famotidine on radiation induced apoptosis in human peripheral blood leukocytes

P. Ghoraeian[1,3], H. Mozdarani[2*], Sh. Akhlaghpoor[1]

[1]Novin Medical Radiation Institute, Tehran, Iran

[2]Department of Medical Genetics, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran

[3]Department of Genetics, Azad Research and Science University, Tehran, Iran

Background: Radioprotective effects of famotidine, an antagonist of H_2 receptor clinically used for peptic ulcer treatment, was previously shown on radiation-induced micronuclei and chromosomal aberration in human peripheral blood lymphocytes and mouse bone marrow cells. This study was conducted to investigate radioprotective property of famotidine against radiation induced apoptosis in human peripheral blood leukocytes. ***Materials and Methods:*** Peripheral blood was obtained from 6 healthy volunteers including three males and three females. 12 µL of blood sample diluted in 1 ml RPMI-1640 supplemented with antibiotics and foetal calf serum was irradiated with a dose of 8 Gy gamma rays generated from a Co-60 source at a dose rate of 1.27 Gy/min. After 48 h incubation in a 37°C incubator, cells embedded in low melting point agarose were transferred to a slide precoated with normal agarose. Cells were lysed and subjected to electrophoresis under neutral condition. Slides were then stained with ethidium bromide and analysed under a fluorescence microscope. 500 cells were analysed for each sample for the presence of apoptosis. The data were statistically evaluated using Man-Whitney non-parametric and ANOVA tests. ***Results:*** Results show a significant increase in apoptosis induction following 8 Gy γ-irradiation comparing with controls ($p<0.001$). The presence of famotidine at 50 and 100 µg/ml did not show any protective effect against radiation induced apoptosis; however, the presence of famotidine at higher concentration (200 µg/ml) significantly deceased radiation induced apoptosis ($p<0.001$). ***Conclusion:*** The obtained results suggest that famotidine suppresses radiation-induced apoptosis at 200 µg/ml, probably via OH radical scavenging and an intracellular antioxidation mechanism. Famotidine appears to be a useful candidate for the future development of post-irradiation radioprotectors. **Iran. J. Radiat. Res., 2005; 3 (1): 31-36**

Keywords: *Leukocytes, apoptosis, famotidine, radioprotection, comet assay.*

INTRODUCTION

Exposing mammalian cells to ionizing radiation activates a genetic program of signalling events that alters the expression and activity of specific genes or proteins. This cascade begins with the genes which are general transcriptional factors and are rapidly induced by growth stimulatory signals. These factors modulate the expression of genes involved in cell cycle progression, repair of DNA damage and apoptosis[1, 2]. This may lead to enhanced survival or cell death depending on which various post irradiation pathways cell follows; also depend on the type and proliferative statue of the cell[3]. The realization that apoptosis may be one of the effective factors in the radiation response of normal tissues and tumours[4-6] has led to a reevaluation of this cell death pathway. Although apoptosis is not the major mode of cell death in irradiated cells in culture[7], some cells such as lymphocytes, male germ cells, thymocytes and epithelial cell lines undergo apoptosis after clinically relevant radiation doses. DNA is the main target in the cell for the biological action of both low and high linear energy transfer (LET) ionizing radiation. The damage can either be induced directly through energy transfer which breaks and distorts DNA strands, or indirectly through radiolysis of surrounding water molecules. This process involves reactive species such as free radicals that reacts with DNA molecule[8, 9]. Ionizing radiation produces a variety of damages in DNA, mainly DNA-DNA crosslink, DNA-Protein crosslink, base damages, single and double strand breaks[10, 11]. Double strand breaks (dsb) are the most critical effects of ionizing radiation leading to chromosomal aberrations and two different modes of cell

Corresponding author:
Dr. Hossein Mozdarani, Department of Medical Genetics, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
Fax: *+98 21 88006544*
E-mail: *mozdarah@modares.ac.ir*

death termed mitotic or clonogenic cell death and apoptosis[12]. A role of DNA damage is suggested in triggering apoptosis, although non–DNA targets such as the cell membrane may be directly or indirectly involved[13].

Apoptosis is a physiological mode of cell death under genetic control[14] characterized morphologically by increased cytoplasmic granularity, cell shrinkage and nuclear and chromosomal condensation, membrane blebbing and the formation of distinctive nuclear bodies[15, 16].

The oxygen radicals produced by low LET ionizing radiation are considered as mediators of apoptosis induction. The OH is generally considered as the most damaging of the oxygen-based free radicals which are produced, and they are believed to account for an estimated 50% of the total damages induced by free radicals[17, 18]. Thus, any agent that could effectively scavenge OH radicals would be considered as potent and important antioxidant.

After the discovery of radioprotective property of cysteine in 1949[19], a vast number of compounds mainly aminothiols were studied for their radioprotective efficacy. The main radioprotective mechanism of these compounds is considered to be radical scavenging; however, they are effective at high doses and produce various side effects. For these reasons, the search for more effective and less toxic radioprotectors has spurred interest in the development of different compounds.

Famotidine, a histamine H_2-receptor antagonist which is usually used for peptic ulcer treatment has been shown to reduce radiation induced DNA damages expressed as chromosomal aberration and micronuclei both *in vivo* and *in vitro*[20-22]. This drug has also been demonstrated to be a highly powerful hydroxyl radical scavenger[23, 24]. Based on these observations the effect of famotidine against radiation induced apoptosis was studied.

Several methods of measuring apoptosis have been developed, mainly based on morphology, others biochemical including the comet assay[25]. Single cell gel electrophoresis (the comet assay) is a reliable and relatively simple method for measuring apoptosis[26-28]. The original comet assay as described by Ostling and Johanson[29] and developed by Singh *et al.*[30, 31] has been further modified to optimize its use for measuring apoptosis[32]. Using this modified neutral comet assay, DNA of apoptotic cells forms structures with diffuse fan-like tails and small heads, whereas normal cells form larger, more defined heads with minimal diffusion[28].

The present study was conducted to examine the effect of famotidine on radiation induced apoptosis in human leukocytes using the neutral comet assay.

MATERIALS AND METHODS

Blood sampling and drug treatment

Peripheral blood samples were obtained by venopuncture from six healthy volunteers including 3 Men (mean age 24.33 ± 5.13 years) and 3 women (mean age 23.67 ± 4.04 years), all non smokers without infectious disease, antibiotic consumption and X-rays during the last month prior to sampling. Fresh blood samples (12 μl) were transferred into a micro tube (Eppendorf) containing 1 ml RPMI-1640 medium (Sigma) supplemented with antibiotics (Penicillin 100 IU/ml, Streptomycin 100 μg/ml), 10% L-Glutamine (2 mM) (Sigma) and 15% fetal bovine serum (FBS, Gibco BRL).

The samples were prepared either in the complete RPMI-1640 medium or medium containing various doses of famotidine. Famotidine powder (provided by Chemodaru Pharmaceutical, Tehran, Iran) was dissolved in RPMI medium at various concentrations (50, 100, 200 μg/ml). The prepared samples, in the presence or absence of famotidine were left in a 37°C incubator for 1 h before gamma irradiation.

Irradiation

Irradiation was carried out with a ^{60}Co γ-ray source (Theratron II, 780 C, Canada) at a dose of 8 Gy. The source to sample distance (SSD) was 80 cm and the dose rate was 1.27 Gy/min. All samples were irradiated on crushed ice, in the presence or absence of famotidine. After irradiation the samples as well as controls were incubated at 37°C for up to 48 h.

Comet assay

Apoptotic and non apoptotic cells in irradiated and control human peripheral blood leukocytes were assessed by the

technique of neutral comet assay[26, 27], with slight modifications. Briefly, the samples were centrifuged for 5 min at 2500 rpm, and then the supernatant was removed. The remainder were mixed with 140 µl of 0.75% low melting point (LMP) agarose (Fermentas) in phosphate buffer saline (PBS). 70 µl of the resulting suspension was applied to each microscope slides window, precoated with a supporting layer of 1% normal melting point (NMP) agarose (Fermentas) in distilled water. The windows were covered with coverslips and kept in 4°C for about 5 min in order to solidify the gel.

Lysis condition

The coverslips were removed and the slides were placed in a dish containing lysis solution, made up with 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris base, 1% N-Lauryl Sarcosine, 1% Triton X-100, 10% dimethyl sulphoxide (DMSO). All materials used for preparation of lysis solution were from Merck Company. The final pH of the lysis solution was about 10.

The slides were left at 4°C in the dark for 30 min, then washed 3 times with electrophoresis buffer (90 mM Tris base, 90 mM Boric acid and 2.5 mM Na2EDTA, pH=8.3-8.4).

Electrophoresis

After lysis, the slides were placed in a submarine horizontal electrophoresis chamber which was filled with fresh electrophoresis buffer. After 15 min electrophoresis at 20 Volts (0.8 V/cm) and 8 mA, the slides were washed with distilled water for 5 min and then fixed in ethanol for 5 minutes at room temperature. The air dried slides were stained with ethidium bromide solution (20 µg/ml) and covered with coverslips.

The number of apoptotic and non apoptotic cells in each slide were analysed using a fluorescent microscope (Nikon) equipped with an excitation filter (510-550 nm) and barrier filter (590 nm), at 200× magnification. Figure 1 shows typical normal and apoptotic cells analysed under microscope. A total number of 500 cells were randomly analysed for each slide and for each sample at one run, at least 1000 cells were analysed.

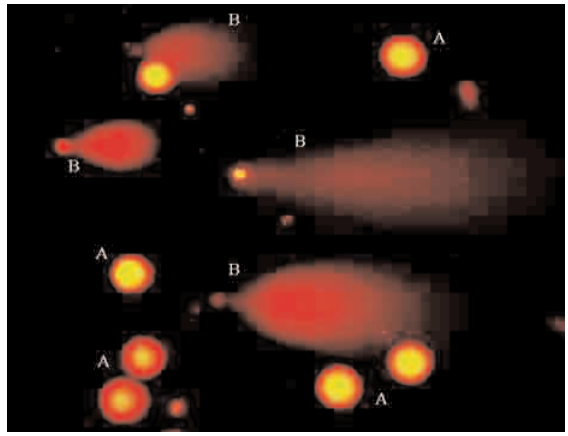

Figure 1. Typical photomicrographs of normal (A) and apoptotic (B) cells analysed under a fluorescent microscope after staining with ethidium bromide. Apoptotic cells are visualised as fan like comet with small head (B).

Statistical analysis

Data were statistically analysed with Man Whitney non-parametric and ANOVA tests using SPSS (version 11.5) software. P-value of less than 0.05 was considered as significant. Because there was no statistical difference between the obtained data for male and female donors, all data were pooled and analysed.

RESULTS AND DISSCUSSION

The results are summarized in table 1 and shown in figure 2. As seen irradiation of whole blood leukocytes with γ-rays led to induction of a relatively high percentage of apoptosis

Table 1. Distribution and frequency of apoptotic cells following gamma irradiation in the presence and absence of various doses of famotidine in human peripheral blood leukocytes irradiated in vitro with a dose of 8 Gy.

Treatment	No. of cells analyzed	No. non-apoptotic cells/sample (Mean ± SE)	No. apoptotic cells/sample (Mean ± SE)
Control	5608	491.4 ± 49.2	69.4 ± 38.5
Famotidine alone			
50 mg/ml	6746	480 ± 45.5	82.5 ± 38
100 mg/ml	7478	539.6 ± 43.1	83.5 ± 50.2
200 mg/ml	6804	486.7 ± 51.6	80.08 ± 41.1
Radiation alone	6493	159.1 ± 56.5	381.5 ± 48.5
Radiation + Famotid			
+ 50 mg/ml	5079	177.2 ± 50.7	330.7 ± 83.3
+ 100 mg/ml	5429	167.9 ± 46.6	375.3 ± 51.6
+ 200 mg/ml	6633	283.6 ± 69.5	269.1 ± 67.7

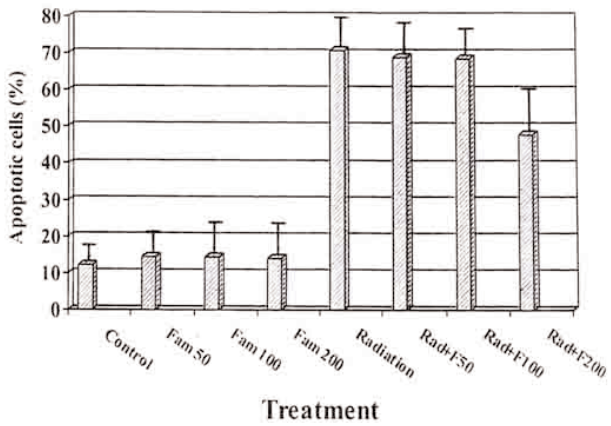

Figure 2. Percentage of radiation induced apoptosis in peripheral blood leukocytes and the effect of famotidine.

compared to the control non-irradiated samples ($p<0.001$). This mode of cell death following irradiation of cells in culture has been shown by several authors for different cell types[4-6, 33-35].

The observation DNA double strand breaks (dsb) trigger apoptosis, as well as dsb and apoptosis linear relation, strongly suggests the involvement of radiation induced DNA damages in apoptosis induction[36]. It is known that sparsely ionizing radiation such as X and gamma rays produce biological effects due to their indirect effects; i.e., free radical formation. A major proportion of the single and double strand breaks in DNA molecules is caused by the formation of hydroxyl radicals[37, 38]. The high increase in apoptosis formation after 8 Gy γ-irradiation during 48 hours might also be due to DNA damages induced by gamma rays (figure 2). It was also shown that OH scavengers play the most effective role in the preservation of the DNA strands against breakage[39]. Various substances were examined for their radioprotective ability against radiation induced apoptosis. For example, suppressive action of WR 2721 (amiphostine) on γ-rays induced apoptosis in mouse bone marrow was found[40]. It was also demonstrated that WR-1065, the active thiol form of amiphostine, is an effective radioprotector of microvascular endothelial cells against radiation induced apoptosis[41]. As shown in figure 2, presence of 200 µg/ml famotidine led to a significant decrease in apoptosis formation following γ-irradiation. Previous studies on this drug, has demonstrated its radioprotective potency against γ-rays induced chromosomal and micronuclei induction[20-22]. Ching *et al.*[23] have shown that histamine H_2 receptor antagonists such as famotidine are in addition to being good inhibitors of histamine-stimulated gastric acid secretion, also highly powerful radical scavenger. It was also shown that famotidine is a powerful scavenger for OH, HOCl, and NH_2Cl with a reaction rate constant of 1.7×10^{10}/mol/s[24]. This rate constant is much higher than the well known hydroxyl radical scavenger manitol (1.7×10^9 /mol/s), and the physiological scavenger of OH, glucose (1×10^9 /mol/s)[23, 24]. Therefore, the reduction in the frequency of radiation induced apoptosis by famotidine, (observed in this study), might be due to its antioxidant and radical scavenging properties (table 1, figure 2). This observation is consistent with findings with other H_2 receptor antagonist, cimetidine, on radiation induced micronuclei[22, 42], as well as apoptosis[42]. It is shown that cimetidine is effective against radiation induced apoptosis via OH radical scavenging and intracellular antioxidation mechanism[33]. There are also other evidences supporting the role of radical scavengers against radiation induced apoptosis. It was recently shown that, treatment of peritoneal resident macrophages of C3H mice with a pharmacological scavenger of superoxide anion, Tiron, significantly suppressed radiation-induced apoptosis[43] and significant decrease in apoptosis in irradiated lymphocytes in the presence of vitamin B1 at concentrations between 1-100 µg/ml[44]. In the present study it was found that doses of 50 µg/ml and 100 µg/ml famotidine were unable to protect peripheral blood leukocytes against radiation induced apoptosis. The interpretation of this finding seems difficult, but it might be due to low concentration of drug present during and after gamma irradiation for being effective against induced DNA damages, hence apoptosis. In other studies with famotidine a dose response relationship was not found (unpublished observations). In conclusion, in line with our previous reports concerning potent radioprotective effect of famotidine against radiation induced cytogenetic damages; this study shows that famotidine might also be a potential drug for reducing radiation induced apoptosis.

ACKNOWLEDGEMENT

The authors wish to express their thanks to Ms Soleimani for irradiation of samples and Mr. Haeri for statistical analysis.

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