



***In vitro* protective effect of atorvastatin against ionizing radiation induced genotoxicity in human lymphocytes**

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Abstract

Atorvastatin (AT) is widely used as a medication for treatment of hypercholesterolemia. Recent studies showed that AT enhanced cell toxicity induced by ionizing radiation in cancerous cells. In this study, the radioprotective effect of AT was investigated against genotoxicity induced by ionizing radiation in human blood lymphocytes. Peripheral blood samples were collected from human volunteers and incubated with AT at different concentrations (0.05, 0.1, 1, or 10 μ M) for two hours. The whole blood was exposed to X-ray at dose 1.5 Gy. Lymphocytes were cultured with mitogenic stimulation to determine the micronuclei in cytokinesis blocked binucleated lymphocyte. AT exhibited a significant decreasing in the frequency of micronuclei in human lymphocytes exposed to ionizing radiation, as compared to with similarly irradiated lymphocytes without AT treatment. The maximum protection and higher decreasing in frequency of micronuclei was observed at 10 μ M of AT (68% decrease), providing maximal protection against ionizing radiation. This data is promising for protection human normal cells from the genetic damage induced by ionizing irradiation.

Key words: Atorvastatin, genotoxicity, radioprotective, micronucleus test.

Introduction

Atorvastatin (AT) is belonging to a class of drugs known as statins. This drug is effective and widely prescribes for the treatment of hypercholesterolemia and the prevention of cardiovascular related diseases. AT acts as a 3-hydroxy-3-methyl-glutaryl co-enzyme A (HMG-CoA) reductase inhibitor, is reducing low-density lipoprotein cholesterol (LDL) levels (1, 2). Recently, several pharmacological properties were proposed for AT such as anti-inflammatory, anti-cancer, anti-oxidant and protective effects (3-6). Several experimental evidences showed that AT exhibited anti-neoplastic effects both in vitro and in animal models (7-10). AT suppressed the growth of cancerous cells and induced apoptosis in various carcinoma cells (11-13). Anti-tumor effect of AT is associated with modulation of cellular MEK/Erk, JNK and pAkt signaling pathways (6, 12, 14, 15). Activation of the autophagy pathway is one of the main mechanism involved in the tumor cell killing effect of AT. Autophagy is a cellular process of degradation of macromolecules and organelles and is activated under oxidative stress. AT is an autophagy inducer; it causes autophagy-associated cell death in prostate cancer cells, through activation of LC3 transcription an autophagosomal marker (14, 16, 17). Prostate cancer cells exposed to gamma irradiation in combination with AT treatment to show significantly reduced colony forming efficiency and an increased number of apoptotic cells. The autophagy pathway may be responsible for apoptosis inducing effect of AT in combination with ionizing radiation on cancer cells (18).

Since AT exhibited anti-cancer effects, its role is

unclear on normal human cells exposed to ionizing radiation. In this study, we investigated the genotoxicity potential induced by of ionizing radiation on human normal lymphocytes, in combination with or without pre-treatment with atorvastatin.

Materials and methods

Blood Treatment

After obtaining permission from research and ethical committees of the Mazandaran University of Medical Sciences, this study was performed. Healthy, non-smoking human volunteers, males men ages between 22 and to 28 years were enrolled in this study. Twelve mL whole blood were collected in heparinized tubes and divided in centrifuge tubes at 0.9mL. Blood samples were treated with 100 μ l solution of atorvastatin (Sobhan Pharmaceutical Company, Rasht, Iran) at the concentrations of 0.05, 0.1, 1, or 10 μ M (final concentration). These samples were incubated for two hours at 37 °C. AT was dissolved in DMSO (Dimethyl sulfoxide, Merck Company, Germany) and diluted in RPMI cultural medium. DMSO concentration was same in control and AT solutions (0.01%).

Ionizing radiation and micronucleus test

Whole blood samples in tubes were irradiated with 6 MeV X-ray beam produced by a radiotherapy machine (Linear accelerator, Siemens, Primus, Germany) at a dose of 150 cGy with a dose rate of 190 cGy/min. Three tubes were used as the control (non-irradiated samples) from three volunteers. After irradiation, subsequently, 0.5 ml of each sample (control and irradiated samples

in duplicate) was added to 4.4 ml of RPMI 1640 culture medium (Gibco, USA), which contained a mixture of 10% fetal calf serum, 100 μ l phytohemagglutinin (Gibco, USA). All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cytochalasin B (Sigma, final concentration: 6 μ l/ml) was added after 44 h of culture. Following 72 h of incubation, the cells were collected by centrifugation for 7 min at 2500 rpm, re-suspended in cold 0.75 M potassium chloride (Merck Company, Germany). Cells were immediately fixed in a fixative solution as of methanol: acetic acid (6:1) (Merck Company, Germany) three times. The fixed cells were dropped onto clean microscopic slides, air-dried and stained with 10% Giemsa (Merck Company, Germany) solution. All slides were evaluated at 100 \times magnification in order to determine the frequency of micronuclei in the cytokinesis-blocked binucleated cells with a well-preserved cytoplasm (19). At each concentration and for each volunteer, 1000 binucleated lymphocyte cells were examined from the irradiated and control cultures in duplicate to record determine the frequency of micronuclei.

Statistical analysis

For each volunteer, at each concentration, the incidence of radiation-induced micronuclei was recorded determined. The data were analyzed with student *t*-test. A probability value of 0.05 was accepted to denote significance.

Results

A typical binucleated lymphocyte with a micronucleus is shown in figure 1. The mean percentage of micronuclei in binucleated lymphocytes in three donors exposed to 1.5 Gy X-ray was 4.80 \pm 0.35, while the percentage in non-irradiated control lymphocytes was 0.48 \pm 0.22. It was showed a statistically significantly ($p < 0.001$) increasing of 10-fold higher in the frequency of micronuclei in samples treated with X-ray (Table 1). The frequency of micronuclei after pre-treatment with AT at doses of 0.05, 0.1, 1, or 10 μ M were 1.78 \pm 0.22, 2.48 \pm 0.14, 2.13 \pm 0.31 and 1.53 \pm 0.25, respectively (Figure 2, Table 1). The data demonstrate that samples incubated with AT, and then exposed in vitro to X-ray, exhibited a significant decrease reducing in the frequency of micronuclei as compared to samples incubated with X-ray alone. Total micronuclei values were reduced by 63%, 48%, 56% and 68% in samples treated with AT and irradiation at concentrations of 0.05, 0.1, 1, or 10 μ M, respectively, compared to irradiated control (Table 1). It was not observed any statistically difference between these each doses of AT in micronuclei frequency.

Discussion

In this study, we showed pre-treatment of human lymphocytes with AT markedly clearly reduced genetic damage induced by ionizing radiation. The frequency of micronuclei in lymphocytes as a marker for of genotoxicity was reduced in irradiated lymphocytes in combination with AT. In 2012, He *et al* reported that AT enhanced cell toxicity induced by ionizing radiation in cancer cell. AT acted as an autophagy inducer, had synergetic ef-

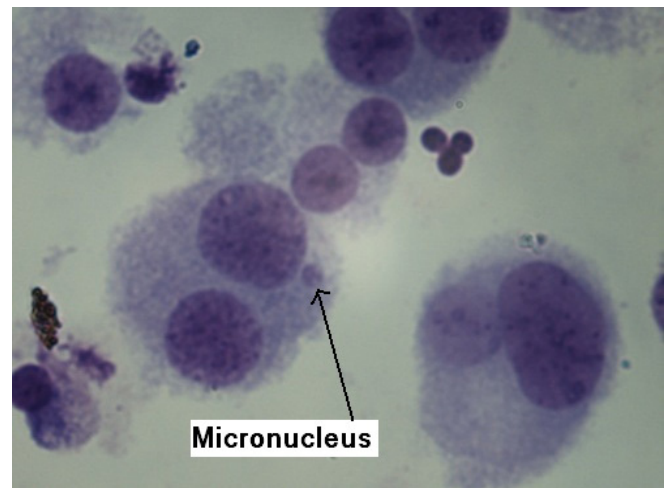


Figure 1. A typical binucleated lymphocyte with micronucleus in our experiments.

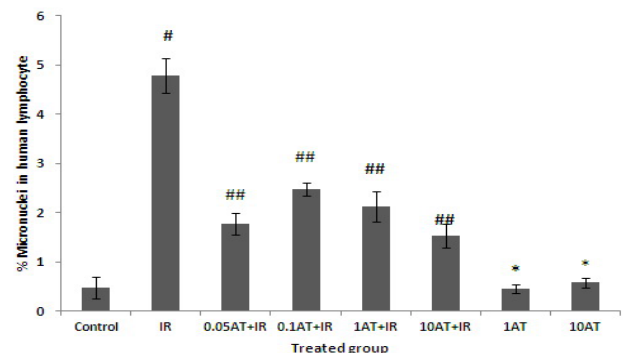


Figure 2. In vitro protection by atorvastatin (AT) at different concentrations (0.05, 0.1, 1 and 10 μ M) against radiation-induced genetic damage induced by X-ray (IR; 1.5 Gy) in cultured whole blood lymphocytes. The data represent average \pm standard deviation of three human volunteers. # $p < 0.05$ compared to control, ## $p < 0.05$ compared to IR, * non-significant compared to control.

fect with ionizing radiation in inducing apoptotic cell deaths and increased the sensitivity of prostate cancer cells to ionizing radiation. Cancerous cells treated with a combination of AT and radiation exhibited a 10%-15% greater number of apoptotic cells in comparison to radiation alone. AT contributes to the radiosensitization of cancerous cells through inducing of autophagy. The blockade of autophagy pathways significantly increased tumor cell survival after irradiation. Autophagy may be the primary pathway that is responsible for AT mediated cell killing and radiosensitization in prostate cancer cells (18). Fritz *et al* showed that lovastatin synthesized cancer HeLa cells to gamma-radiation-induced cell killing. This sensitizing effect was related to abrogation of G2 blockage and a concomitant increase in apoptotic/necrotic cell death(20). In contrast to the radiosensitive effect of AT, several studies reported the protective effects of AT on normal cells against toxicity caused by oxidative stress. AT had a protective effect on the myocardial injury induced by oxidative stress in rats. AT increased antioxidant enzymes such as superoxide dismutase (SOD) activity and while it decreased oxidative marker such as malondialdehyde (MDA)(21). Renoprotective effect of AT was evaluated against streptozotocin (STZ)-induced kidney injury in rats. AT increased SOD and glutathione peroxidase (GSHPx) activities, while the MDA content was reduced. Then AT enhanced an-

Table 1. The frequency of micronuclei induced *in vitro* by 150 cGy X-ray radiation (IR) in cultured blood lymphocytes from human volunteers examined treated at different doses of atorvastatin (AT).

Group	% Micronuclei in binucleated human lymphocyte			
	I	II	III	Mean± SD
Control	0.49	0.69	0.25	0.48±0.22
IR	4.4	4.92	5.07	4.80±0.35 [#]
0.05AT+IR	1.8	1.99	1.55	1.78±0.22 ^{##}
0.1AT+IR	2.33	2.61	2.487	2.48±0.14 ^{##}
1AT+IR	1.79	2.4	2.19	2.13±0.31 ^{##}
10AT+IR	1.3	1.5	1.8	1.53±0.25 ^{##}
1AT	0.49	0.36	0.53	0.46±0.09 [*]
10AT	0.56	0.68	0.5	0.58±0.09 [*]

1000 BN cells were examined in each sample.

p<0.05 compared to control, ##p<0.05 compared to IR, *non-significant compared to control (Student T-Test was applied for comparison of data).

tioxidant capacity in kidney tissue (22). AT ameliorated contrast medium induced medullary damage, restored renal function, and suppressed renal tubular apoptosis by inhibiting the Rho-associated protein kinase 1 pathway. AT up-regulated the expression of Bcl-2, down-regulated the expression of Bax (23). Nubel et al showed that lovastatin as a statin medication reduced cell proliferation and DNA damage induced by ionizing radiation on primary human umbilical vein endothelial cells. Lovastatin attenuated the increase in p53/p21 protein level and impaired the activation of nuclear factor-kappaB, Chk-1, and Akt kinase but did not inhibit extracellular signal-regulated kinase activation (24). AT had protective effects on liver, kidney, heart injuries and DNA damage induced by doxorubicin, via antioxidant, anti-nitrosative, anti-inflammatory and anti-apoptotic mechanisms (3, 25). These results show that AT contributes on protective effect against toxicity caused by oxidative stress in normal tissues through mainly antioxidant and anti-inflammatory effects (26-28). In this study we showed that AT mitigated DNA damage induced by ionizing radiation in human lymphocytes. It is well documented that free radicals and inflammation are main reasons for DNA damage induced by ionizing radiation. Normal cell is going to dysfunction in inflammatory process induced by ionizing radiation (29). It is cleared that antioxidant activity, anti-inflammatory effect, and modulation of cell signaling pathways are main proposed mechanisms for radioprotective effects of AT against *in vitro* genotoxicity induced by ionizing radiation on lymphocytes.

The radioprotective effect of AT is probably related to antioxidant and anti-inflammatory properties of AT on human lymphocytes. Although AT sensitized cancerous cell to ionizing radiation, it likely protects, *in vitro*, normal cell against DNA damage induced by ionizing radiation. This result provides a new indication of atorvastatin for protection of normal cells exposed to ionizing radiation and it is probably promising in treatment of cancer patients during radiation therapy.

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