Protective Effect of Atorvastatin on Radiation-induced Vascular Endothelial Cell Injury \textit{In Vitro}

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Vascular endothelial cells are very sensitive to ionizing radiation, and it is important to develop effective preventive agents and measures in radiation exposure protection. In the present study, the protective effects of atorvastatin on irradiated human umbilical vein endothelial cells (HUVEC) and the possible mechanisms were explored. Cultured HUVEC were treated by atorvastatin at a final concentration of 10 μmol/ml for 10 minutes, and then irradiated at a dose of 2 Gy or 25 Gy. Twenty-four hours after irradiation, apoptosis of HUVEC was monitored by flow cytometry, and the expression of thrombomodulin (TM) and protein C activation in HUVEC was respectively assessed by flow cytometry and spectrophotometry. After treatment with atorvastatin for 24 h, the rate of cell apoptosis decreased by 6% and 16% in cells irradiated with 2 Gy and 25 Gy, respectively. TM expression increased by 77%, 59%, and 61% in untreated cells, 2 Gy irradiation-treated cells, and 25 Gy irradiation-treated cells, respectively. The protein C levels in 2 Gy and 25 Gy irradiation-treated cells were reduced by 23% and 34% when compared with untreated cells, but up-regulated by 79% and 76% when compared with cells which were irradiated and treated with atorvastatin. In conclusion, these data indicate that atorvastatin exerts protective effects on irradiated HUVEC by reducing apoptosis by up-regulating TM expression and enhancing protein C activation in irradiated HUVEC.

INTRODUCTION

Vascular endothelial cells (VEC) are very sensitive to ionizing radiation, and data from research on radiation damage to the intestinal tract, lungs, and central nervous system show that VEC are the first tissue type damaged by irradiation.\textsuperscript{1–6} Paris et al.\textsuperscript{7} confirmed that apoptosis of VEC is the initial pathologic change in intestinal radiation damage. Thus, finding ways to protect VEC and developing effective preventive agents are important goals in protection and treatment from the effects of radiation exposure. Istvan et al.\textsuperscript{8} reported that hydroxymethyl-coenzyme A (HMG-CoA) reductase inhibitors can effectively inhibit the conversion of HMG-CoA to methyl valerate, and can thereby block cholesterol synthesis and reduce the generation of low-density lipoprotein. Moreover, statins have been shown to reduce inflammatory responses, stabilize cell metabolism, improve blood circulation, and decrease the oxidative damage of endothelial cells.\textsuperscript{9–12} Fluvastatin has a protective effect on radiation-induced lung damage, but the mechanism remains to be clarified.\textsuperscript{13}

Thrombomodulin (TM), a thrombin membrane receptor on the surface of VEC, activates protein C by combining with thrombin. Once activated, protein C can act as an anticoagulant by accelerating the inactivation of factors Va and VIIIa.\textsuperscript{14} TM has also been reported to have anti-inflammatory activity by inhibiting the secretion of the monocyte pro-inflammatory factors, TNF and IL-1, which in turn effectively alleviate adhesion of leukocytes to VEC and reduce the injury of VEC.\textsuperscript{15,16} After irradiation, the expression of TM in VEC is significantly decreased.\textsuperscript{2–4} Taken together, there is accumulating data that shows TM and protein C play an important role in maintaining the normal function of VEC. In the present study, whether or not atorvastatin has a protective effect on human umbilical vein endothelial cells...
(HUVEC) was determined and the role of TM and protein C activation in this process was elucidated.

MATERIALS AND METHODS

Endothelial cell culture

HUVEC and endothelial cell culture medium (EGM-2) were purchased from Cambrex Life Sciences Company (Baltimore, MD, USA). EGM-2 consists of 500 ml of DMEM culture medium, 0.5 ml of 10 μg/ml recombinant human epithelial cell growth factor, 0.2 ml of 1 mg/ml of hydrocortisone, 0.5 ml of 10 μg/ml of vascular endothelial cell growth factor, 2 ml of 3 mg/ml of bovine brain extract, 10 ml of pregnant bovine serum, 0.5 ml of 50 mg/ml of gentamicin, and 50 μg/ml of amphotericin B.

Cell exposure

One hour before irradiation, the cell culture medium was changed and 10 μ mol/ml of atorvastatin (Japan Vakuf Fine Chemicals Corporation, Japan) was added. The endothelial cells were then placed into a DOT-7A radiator with 137Cs radioactive sources to receive 2 Gy or 25 Gy γ-ray irradiation, with a dose rate of 285.7 cGy/min. Twenty-four h after irradiation, morphologic changes of irradiated HUVEC were observed under an inverted microscope. The cells were collected for the following studies.

Detection by flow cytometry

Cell surface antigen expression, cell cycle status, and apoptosis were studied using flow cytometry. TM was stained with PE-labeled anti-human TM monoclonal antibody (CD141), tissue factor anti-vascular endothelial cell activated tissue factor antibody (CD142) marker, and the negative control was stained using PE-labeled mouse IGG1 homologous proteins. Cell apoptosis was studied using 7-aminoactinomycin D (7-AAD) staining. Antibodies and QuantiBrite antigen calibration beads were purchased from Becton Dickinson Life Sciences Limited. At a corresponding time point, 10^7 cells were measured. At the same time, PE-labeled antigen QuantiBrite calibration beads were purchased from Becton Dickinson Life Sciences Limited. At a corresponding time point, 10^7 cells were measured. At the same time, PE-labeled antigen QuantiBrite calibration beads were purchased.

Protein C activation detection

TM function was reflected by measuring the activation capacity of protein C in the cultured HUVEC. A combination of endothelial cell surface TM and thrombin-enabled protein C was activated so that the activated protein C decomposed its substrate to bring about a coloration reaction. The coloration was measured spectrophotometrically using a specific wavelength representing the activity of protein C. Therefore, it reflects the function of the endothelial cell surface TM. Five d thereafter, the endothelial cells were cultured in 96-pore plates, and washed with EDTA/PBS. Thrombin (0.1 unit) and protein C (60 μl) were added to a final concentration of 500 n mol/L, and the reaction was carried out at 37°C for 60 min to generate APC. Then, hirudin, at a final concentration of 0.05 unit/μl, was added to terminate the reaction. Color substrate S-2366 was added using a micro-plate spectrophotometer to measure the optical density at 405 nm. Absorption was measured every 5 min for 60 min. The results are expressed as the average slope.

Statistics

The experimental results are expressed as the mean ± standard tolerances. SPSS 13.0 software was used to compare experimental data with the multiple comparison (LSD) method and factorial design analysis of variance, in which a P < 0.05 denotes a significant difference, and a P < 0.01 is an extremely significant difference.

RESULTS

Morphologic change of VEC irradiation by atorvastatin treatment

Normal HUVEC form a monolayer of cobblestone-like oval cells in close contact. After treatment by atorvastatin for 24 h, HUVEC assumed shuttle, triangular, or irregular shapes. There were many pseudopodia around the cells and more intercellular gaps, indicating that cells were in a relative active state of motion. When compared with HUVEC which had only been irradiated, pseudopodia were more obvious, but with less pyknotic and necrotic cells than HUVEC which had been irradiated and treated with atorvastatin (Fig. 1). The number of HUVEC decreased significantly after irradiation for 24 h, and was significantly less that in cells which had been irradiated and treated with atorvastatin.

Effect of atorvastatin treatment on the HUVEC apoptosis

The rate of apoptosis of normal HUVEC was nearly 9%, and increased in a dose-dependent manner following irradiation; specifically, the rate was 13% and 23% in HUVEC irradiated with 2 Gy and 25 Gy, respectively. After treatment with atorvastatin, the rate of apoptosis did not demonstrate a significant difference among non-irradiated cells, and cells irradiated with 2 Gy and 25 Gy. However, the rate of apoptosis of HUVEC which were irradiated and treated with atorvastatin treatment was 3%, 6%, and 16% lower than cells which were non-irradiated, and irradiated with 2 Gy and 25 Gy, respectively (t = 8.027, 4.178, and 17.863, respectively; P < 0.01; Fig. 2).
Effect of atorvastatin treatment on quantity and function of TM expression

After being treated by atorvastatin for 24 h, the surface antigen binding site of TM in non-irradiated HUVEC that can reflect the level of expression of TM increased rapidly by 77% when compared with normal HUVEC \((t = 27.395, P < 0.01)\), while it did not change significantly in HUVEC irradiated with 2 Gy and 25 Gy. However, the level of expression of TM increased by 59% in HUVEC which were irradiated with 2 Gy and treated with atorvastatin compared with HUVEC which were only irradiated with 2 Gy \((t = 26.420, P < 0.01)\). The level of expression of TM increased by 61% in HUVEC which were irradiated with 25 Gy and treated with atorvastatin when compared with HUVEC which were only treated with 25 Gy irradiation \((t = 58.065, P < 0.01)\).

The difference in the level of expression of TM between normal HUVEC and HUVEC which were irradiated with 2 Gy and treated with atorvastatin was statistically significant \((t = 21.605 \text{ and } 22.284, \text{ respectively}; P < 0.01; \text{ Fig. 3})\).
Effect of atorvastatin treatment on tissue factor expression

After irradiation with 2 Gy and 25 Gy, the tissue factor expression in HUVEC decreased slightly in a dose-dependent manner, and the expression of tissue factor decreased (1618 / cell in the 0-Gy group, 1178 in the 2-Gy group, and 774 in the 25-Gy group). The level of expression of tissue factor in HUVEC which were irradiated with 25 Gy was significantly higher in comparison with their corresponding dose group, a: t values were 8.027, 4.178, and 17.863, respectively; P < 0.01).

Fig. 3. Effect of atorvastatin on irradiated HUVEC-TM expression. (A: A presentive typical feature of HUVEC-TM expression after a 25 Gy irradiation. B: A typical feature of HUVEC-TM expression when HUVEC received a 25 Gy irradiation and atorvastatin treatment. C: Statistical analysis of HUVEC-TM expression when they received different dose of irradiation with or without atorvastatin treatment. in comparison with their corresponding dose group, a: t values were 27.395, 26.420, and 58.065, respectively; P < 0.01).
lower than non-irradiated HUVEC. After treatment with atorvastatin, the level of expression of tissue factor in HUVEC showed no significant change when compared with HUVEC which were not treated with atorvastatin. However, the level of expression in HUVEC which were irradiated with 25 Gy was still lower than the HUVEC which were not irradiated and treated with atorvastatin (\(t = 3.108; P < 0.01\) vs. 0-Gy + A group; \(t = 5.227, P < 0.01\) vs. 0-Gy group).

**Fig. 4.** Effect of atorvastatin and irradiation on HUVEC tissue factor. (A: A typical feature of HUVEC-tissue factor expression after a 25 Gy irradiation. B: A typical feature of HUVEC-tissue factor expression when HUVEC received a 25 Gy irradiation and atorvastatin treatment. C: Statistical analysis of HUVEC-tissue factor expression when they received different dose of irradiation with or without atorvastatin treatment. There were no significant differences in the two groups with or without atorvastatin treatment at the same irradiation dose. a: \(t = 3.108, P < 0.01\) vs. 0-Gy + A group; b: \(t = 5.227, P < 0.01\) vs. 0-Gy group).

**Effect of atorvastatin treatment on activated protein C in HUVEC**

TM function in HUVEC was assessed by examination of activated protein C. The content of activated protein C in the non-irradiated HUVEC increased significantly after atorvastatin treatment. After irradiation at a dose of 2 Gy, the content of activated protein C decreased significantly when compared with normal HUVEC, and increased rapidly by 79% when treated with atorvastatin (Fig. 5). The content of activated protein C in HUVEC which were irradiated with 25 Gy demonstrated a similar trend (Fig. 6).

**DISCUSSION**

VEC, which are relatively sensitive to ionizing radiation, play an important role in the pathologic process of radiation damage. Accordingly, it is of extreme significance to identify effective protective measures and treatment of VEC radiation damage.\(^3,6\) The present study showed that cell apoptosis occurs in HUVEC after \(\gamma\)-ray irradiation at various doses, and the rate of apoptosis increases with the increment of the dose of irradiation. However, after irradiation with an identical dose, the incidence of VEC apoptosis was reduced significantly by the action of atorvastatin, showing that atorvastatin can effectively protect VEC damage induced by ionizing radiation.
Statins, currently the preferred anti-lipemic agents, have several advantages, such as safety, effectiveness, remarkable tolerability, and a long half-life, and duration of action. In this regard, remarkable benefits have been achieved in the prevention and treatment of cardiovascular diseases. A recent study has shown that statins also have multiple effects of regulating cardiovascular endothelial metabolism, improving cardiovascular endothelial function, anti-inflammatory, and antithrombotic action.\textsuperscript{3,4,11} Statins have been used in clinical trials and tested in the protection and treatment of kidney and liver diseases, and are also used as agents for improvement of effectiveness of tumor radiotherapy,\textsuperscript{12–19} but there are few reports concerning the use of statins in protecting and treating acute radiation damage. Indeed, atorvastatin can effectively reduce endothelial cell apoptosis caused by radiation.

The possible mechanisms of the protective effect of atorvastatin were explored in the present study. It was found that TM expression was up-regulated in irradiated HUVEC that accepted atorvastatin administration when compared with those without atorvastatin administration. Given the important role of TM in the function of VEC, it is reasonable to draw the conclusion that TM is the main target of atorvastatin with respect to the protective effect of irradiated HUVEC.

Our previous study showed that TM expression was significantly increased only 8 h after VEC was incubated, reaching a climax at 24 h. The direct effect of ionizing radiation on cellular DNA damage was an acute event. Thus, it is speculated that TM increment does not have effect on radiated cell DNA damage. This study showed that statins can increase TM expression of irradiated VEC, and the increased TM is involved in activating protein C. TM is the thrombin membrane receptor of the VEC surface, which activates protein C by way of combining with thrombin. Activated protein C, with function of anticoagulant, can accelerate inactivated effect on factors Va and VIIIa, and increase catalytic activity restraining on fibrinolysis inhibitor activated by thrombin.\textsuperscript{[14]} van de Wouwer et al.\textsuperscript{15,16} reported that TM has anti-inflammatory function, which can inhibit secretion of monocyte pro-inflammatory factor TNF and IL-1 so that the number of endothelial cell adhesion molecule ICAM-1 and leukocyte adhesion molecule are reduced, thus effectively alleviate adhesion of leukocyte and endothelial cell. Research by Wang et al.\textsuperscript{2–4} showed that surface TM expression of irradiated-injured tissue endothelial cell decreases significantly. Although the mechanism of effects of TM in protection of the endothelial cells in acute radiation injury is not clear, it undoubtedly plays a very important role in this regard when it is viewed from the whole tissue and aspects of the development of state of the disease. As statins have a different affinity on HMG-CoA reductase, intensity of effect will be somewhat different. This can be explained in the experiment that atorvastatin has different effects on improvement of endothelial cell TM.

To summarize, this study showed that atorvastatin can effectively prevent the γ-ray irradiation damage to VEC. This protective function is related to changing VEC cycle distribution by statins and strengthening its TM expression and protein C activating capacity. At the same time, it prompts that statins may have effective effects in protection of irradiation damage and healing of radioactive injury wound. The mechanism underlying the protective effect of statins on VEC and the overall protective effects merits further study.

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