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γ-Irradiation Induced Adhesion Molecules are Reduced by Vitamin C in Human Endothelial Cells

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Abstract – Inflammation is a frequent radiation-induced reaction following therapeutic irradiation. Treatment of human umbilical endothelial cells (HUVEC) with γ-irradiation (γIR) induces the expression of adhesion proteins such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. Since the upregulation of these proteins on endothelial cell surface has been known to be associated with inflammation, interfering with the expression of adhesion molecules is an important therapeutic target. In the present study, we demonstrate that vitamin C inhibits γIR induced expression of ICAM-1, VCAM-1, and E-selectin on HUVEC in a dose- and time dependent manner. Vitamin C also inhibited the production of Nitric oxide (NO) induced by γIR. These data suggest that vitamin C has therapeutic potential for the treatment of various inflammatory disorder associated with an increase of endothelial leukocyte adhesion molecules.

Keywords □ Vitamin C, γ-irradiation, Endothelial cells, ICAM-1, VCAM-1, E-selectin, NO

INTRODUCTION

Vitamin C is an essential vitamin for humans, primates, guinea pigs, and few other animals and insects that lack the enzyme L-gulono-lactone oxidase, the final enzyme in the biosynthetic pathway for vitamin C (Nishikimi and Yagi, 1991; Nishikimi, *et al.*, 1994). Vitamin C is a strong antioxidant that sustains a balance of reactive oxygen species(ROS) generated in the course of aerobic ATP generations (Frei *et al.*, 1989; Strain and Benzie, 1999), inhibits cell death, and prevents mutations induced by ROS (Guaiquil *et al.*, 2001; Lutsenko *et al.*, 2002). Additionally, there is literature pointing to the role of vitamin C in enhanced host defense and the modulation of inflammatory reactions (Heuser and Vojdani, 1997; William *et al.*, 1984; Nowak *et al.* 1989).

Ionizing radiation damage is partially characterized by the generation and the maintenance of an inflammatory reaction (Hruza and Pentland, 1993). An important event in this inflammatory response is the localization of leukocytes at the sites of inflammatory lesions through a multistep process. The endothelial cell adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), play an important role in leukocyte adhesion and transendothelial migration at sites of inflammation. Endothelial expression of these molecules has been known to be elevated in both a temporal and spatial association with inflammatory cell infiltrates (Carlos and Harlan, 1994). When activated by inflammatory cytokines, endothelial cells exhibit an upregulation of specific adhesion molecules on their surface, the ligands for which are borne on circulation leukocyte (Bevilacqua *et al.* 1985; Springer *et al.*, 1990). A logical target for new drug development would be the design of compounds that would interfere with adhesion molecule interactions. It has been suggested that various small molecules including glucocorticoids, aspirin and pentoxifylline inhibit the upregulation of adhesion protein expression and have a protective effect on inflammatory diseases (Brostjan *et al.*, 1997; Weber *et al.*, 1995; Neuner *et al.*, 1997).

Nitric oxide (NO) is synthesized by many cell types in various tissues and it acts as a vascular relaxing agent, a neurotransmitter and an inhibitor of platelet aggregation (Moncada *et al.*, 1991; Loncoln *et al.*, 1997). In addition, NO is generated dur-

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ing immune and inflammatory responses (Moncada *et al.*, 1991; Loncoln *et al.*, 1997). NO may induce toxic reactions against other tissue of the host and since it is generated at high levels in certain types of inflammation, it has been implicated as pro-inflammatory agent. Equally, it may act as an anti-inflammatory or immunosuppressive agent via its inhibitory or apoptotic effects on cells. However, there has been little reported that has assessed the roles of NO in γ-irradiation-induced inflammation.

Since vitamin C has modulatory activity of inflammation and NO play an important role in inflammation, we determined whether vitamin C modulates the expression of adhesion proteins and NO release in irradiated-human umbilical vein endothelial cells (HUVEC). The results of the present study suggest that vitamin C inhibits the upregulation of γIR-induced adhesion protein expression. We also observed that vitamin C blocked the production of NO induced by γIR.

MATERIALS AND METHODS

Reagents

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Anti-ICAM-1 (BBA3), anti-VCAM-1 (BBA6) and anti-E-selectin (BBA1) antibodies were purchased from R & D Systems, USA. Anti-mouse IgG-HRP and p-nitorphenyl phosphate were purchased from Sigma Chemical Co. Fetal bovine serum was purchased from Gibco, USA.

Cells and Cell culture

HUVECs were purchased from Clonetics (San Diego, CA) and were grown in EGM-2 medium (Clonetics) in gelatin coated tissue culture flasks. For subculturing, the cells were detached using 0.125% trypsin containing 0.01 M EDTA. Cells used in this study were from the first to third passage.

γ-irradiation on cells

Cells were irradiated by a previous method of Gaugler *et al.* (1997). Just before irradiation, the medium of confluent cells was changed and replaced with new medium. The cells were then uniformly irradiated at room temperature with various doses of a ^{137}Cs γ-source (dose rate of 5.94 Gy/min) (IBL 437 C type H, CIS biointernational, France). The culture medium was renewed after irradiation. For each dose, control cells were simultaneously exposed to sham irradiation.

Cytotoxicity evaluation

Vitamin C and γIR at concentrations presented herein were shown to be non-toxic by the following procedure. Viability of HUVEC treated with vitamin C and/or γIR was determined using MTT assay. Irraditated HUVECs were cultured in gelatin coated 96-well microplate (Costar Products, Cambridge, MA) until confluent. Cells were treated with or without vitamin C in quadruplicate for indicated time and subsequently 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) was added for 4 h. In addition, a visual inspection of the cells was performed by trypan blue exclusion staining under inverted microscope.

ELISA for measurement of adhesion molecules

The cell surface expression of adhesion molecules on endothelial monolayers was quantified using ELISA by modification of the methods described previously (Gupta and Ghosh, 1999). After irradiation, HUVECs were seeded at a concentration of 2×10^4 cells/well in 96-well, flat bottom, gelatin-coated plates (Costar Products). The cells were incubated without or with various doses of vitamin C for the time indicated in the text to measure ICAM-1, VCAM-1 and E-selectin expression. Following incubation, the cells were washed with phosphate buffer saline pH 7.4 (PBS) and fixed with 10% glutaraldehyde for 30 min at 4°C. Bovine serum albumin (1.0% in PBS) was added to the cells to reduce non-specific binding. Cells were incubated with anti-ICAM-1, anti-VCAM-1 and anti-E-selectin monoclonal antibody or isotype matched control antibody (0.25 g/ml, diluted in blocking buffer) overnight at 4°C washed with PBS followed by incubation with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1 g/ml, diluted in PBS). The cells were then washed with PBS and exposed to the peroxidase substrate (p-nitorphenyl phosphate 1 mg/ml in 0.1 M glycin buffer, pH 10.4 containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$). Absorbance was determined at 405 nm by a Molecular device microplate reader (Menlo Park, CA, USA). The absorbance values of isotype matched control antibody were taken as blank and subtracted from the experimental values.

Nitrite determination

Irradiated HUVECs were treated with various doses of vitamin C for times indicated in the text and the accumulation of nitrite in culture supernatant was measured using the assay system described by Ding *et al.* (1998). Briefly, 100 μl supernatant was removed from each well into an empty 96-well plate. After the addition of 100 μl Griess reagent to each well, absorbance

at 540 nm was measured using an a Molecular device microplate reader. Nitrite concentration was calculated from a $NaNO_2$ standard curve. The levels of absorbance are indicative of NO production. Griess reagent was prepared by mixing one part 0.1% naphthylethylenediamine dihydrochloride in distilled water plus one part 1% sulfanilamide in 5% concentrated H_3PO_4.

Statistical Anaylsis

Each data were given as means±S.E.M. Statistical difference between groups was determined by one-way analysis of variance (ANOVA) and significant values were represented by an asterisk (*$p<0.05$, **$p<0.01$).

RESULTS

Endothelial cell viability and growth after γIR

Although the effect of radiation on endothelial cells has been extensively reported (Rubin *et al.* 1989; Eissner *et al.* 1995; Gaugler *et al.* 1997), the radiosensitivity of HUVEC was assessed in our culture conditions. Viability of the adherent endothelial cells was >95% at the different times tested after exposure. However, the number of irradiated cells was decreased with time after exposure, while the number of non-irradiated cells was stable. Three days after a 8-Gy irradiation, the number of irradiated cells represented 83% of the number of control cells. This percentage was decreased to 10% by 5 days after irradiation (Fig. 1). In addition, when the cells were treated with various doses of irradiation, the expression of ICAM-1 was maximally induced at 8-Gy irradiation (data not shown). Therefore, in subsequent studies 8-Gy irradiation was used.

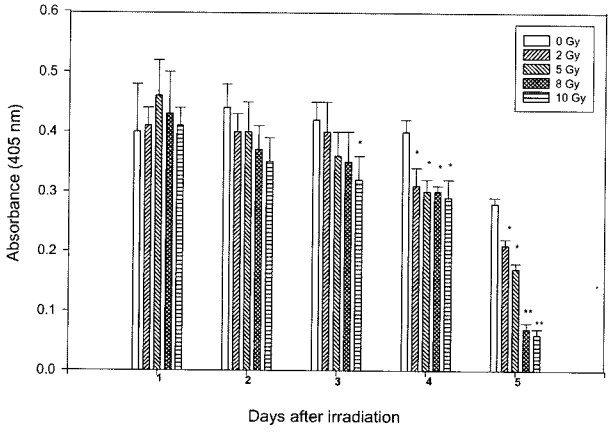

Fig. 1. Effect of γ-irradiation on cell viability. Cells were irradiated with various doses of γ-ray for various times. Each experiment was performed in quadruplicate. The results are mean ± S.E.M from three independent experiments. *$p<0.05$, **$p<0.01$; significantly different from control (sham-irradiated cells).

Dose dependent inhibition of γ-irradiation induced ICAM-1 expression by vitamin C

To examine the effect of vitamin C, HUVECs were incubated without or with various concentrations of vitamin C for 3 days after γIR. The time of incubation and concentration of vitamin C used in these experiments had no effect on the viability as determined by trypan blue staining and morphology of the endothelial cells (data not shown). These concentrations are in complete agreement with the optimum concentrations of vitamin C needed in previous studies (Guaiquil *et al.*, 2001). As detected by ELISA, ICAM-1 was expressed at low levels on unstimulated endothelial cells and was significantly induced by γIR (Fig. 2). Vitamin C did not have effect on the basal level of ICAM-1 expression, whereas it led to a reduction in the γIR-induced ICAM-1 expression in a dose dependent manner (Fig. 2).

Effect of vitamin C on γ-irradiation induced VCAM-1 and E-selectin expression by HUVEC

In addition to ICAM-1, γIR also induced VCAM-1 and E-selectin expression in endothelial cells. To examine the effect of vitamin C on γIR-induced expression of E-selectin and VCAM-1, HUVECs were incubated with various concentrations of vitamin C for 6 h for the expression of E-selectin and for 3 days

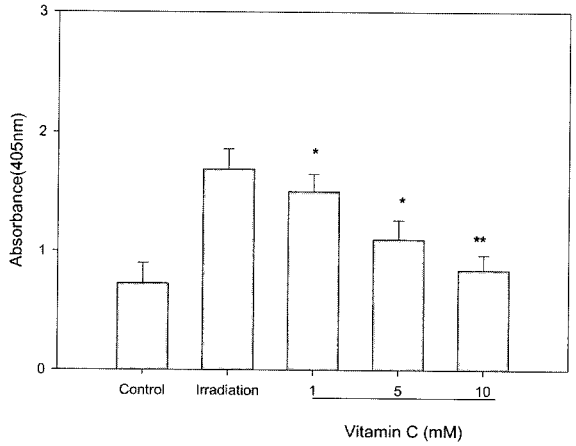

Fig. 2. Dose dependent inhibition of γ-irradiation induced ICAM-1 expression by vitamin C. Three independent experiments were performed in which confluent HUVECs were sham- or 8 Gy-irradiated without or with indicated concentrations of vitamin C and the expression of ICAM-1 measured by ELISA. The results are mean±S.E.M from three independent experiments. *$p<0.05$, **$p<0.01$; significantly different from irradiation-treated control.

for VCAM-1, respectively, As measured by ELISA, the unstimulated cells expressed detectable amounts of E-selectin and VCAM-1 (Fig. 3). Upon induction with γIR, a significant increase in the expression of E-selectin was observed. Treatment with vitamin C inhibited significantly the expression of VCAM-1 induced by γIR in a dose dependent manner (Fig. 3A). Similarly, γIR-induced E-selectin expression was also inhibited by vitamin C (Fig. 3B). Taken together, these results suggest that vitamin C is effective in blocking the induced level of expression of ICAM-1, VCAM-1 and E-selectin.

Inhibition of γIR-induced NO production by Vitamin C

Since NO has been known to be an important modulator of the inflammatory response to various stimuli, we determined the effect of vitamin C on NO production in endothelial cells.

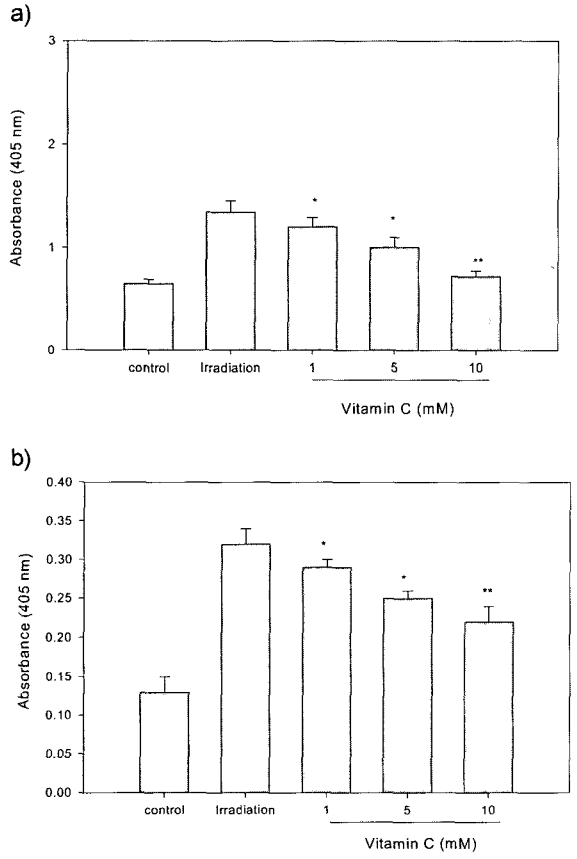

Fig. 3. Effect of vitamin C on γ-irradiation induced VCAM-1 (a) and E-selectin (b) expression by HUVEC. Three independent experiments were performed in which confluent HUVECs were sham- or 8 Gy-irradiated without or with indicated concentrations of vitamin C and the expression of VCAM-1 or E-selectin measured by ELISA. The results are mean ± S.E.M from three independent experiments. *p<0.05, **p<0.01; significantly different from irradiation-treated control.

As shown in Fig. 4, treatment of HUVEC with γIR results in an increase of NO release that was inhibited by vitamin C in a dose dependent manner.

DISCUSSION

Vitamin C has been suggested as having both a preventative and therapeutic role in a number of pathologies including inflammtory reaction (William *et al.*, 1984; Nowak *et al.* 1989). Although vitamin C is found to have anti-inflammatory properties, very little is known in regard to its effect on the induction of cell adhesion molecules by γIR. In the present study, vitamin C blocked γIR-induced expression of leukocyte adhesion molecules, ICAM-1, VCAM-1 and E-selectin. Thus, vitamin C possesses anti-inflammatory effect on the expression of adhesion protein induced by radiation. This compound also inhibited the NO production in γ-irradiated HUVEC.

Radiation has been shown to induce the expression of a number of genes that participate in the inflammatory response. These include TNF-α and IL-1 which have been known to induce the expression of adhesion molecules such as E-selection when added to endothelial cells in culture (Sherman *et al.*, 1991; Brach *et al.*, 1993; Bevilacqua *et al.*, 1989). When the effect of γIR on cytokine production was examined in HUVEC, it did not induce the production of TNF-α in irradiated cells (data not shown). Recently, Hallahan *et al.* (1995) demon-

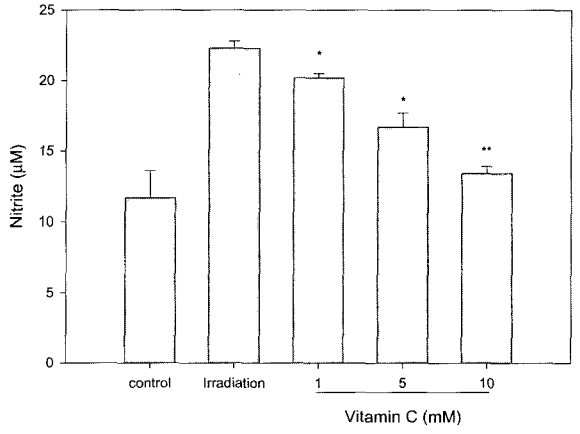

Fig. 4. Effect of vitamin C on γ-irradiation induced NO production in HUVEC. Three independent experiments were performed in which confluent HUVECs were sham- or 8 Gy-irradiated without or with indicated concentrations of vitamin C. The conditioned medium was collected, and the nitrite concentration was determined by means of the Griess reagent. The results are mean ± S.E.M from three independent experiments. *p<0.05, **p<0.01; significantly different from irradiation-treated control.

strated that E-selectin gene induction by ionizing radiation is independent of cytokine induction. In accordance with their report, our data confirmed that adhesion protein expression did not require cytokine synthesis.

Nitric oxide is a biologically active gas that is synthesized by a variety of cells, including vascular endothelium, from the guanido group of L-arginine. This reactive nitrogen molecule has been invoked as a mediator of vascular phenomena such as arteriolar dilation, platelet aggregation, and platelet-leukocyte adhesion (Moncada, 1992). It has been suggested that NO is able to inhibit LPS-induced ICAM-1 expression (Spiecker *et al.*, 1998). In addition, Kawachi *et al.* (1999) demonstrated that iNOS-/- mice injected with TNF-α showed an enhancement of VCAM-1 expression in 50% of all tissues compared to wild-type controls. Based on these findings NO could inhibit the expression of adhesion molecules. Our data showed that γIR induces the production of NO and vitamin C inhibits NO release. Recently, it has been shown that UVB radiation acts as a potent stimulator of NO in human endothelial cells and NO is involved in skin erythema and inflammation (Deliconstantinos *et al.*, 1996). Thus, a role could be proposed for NO either in inhibiting or promoting inflammation. At present the precise mechanisms accounting for these modulation are unknown. It may be possible, however, to override inhibiting by enhancing inflammation. Moreover, the involvement of NO in the modulation and regulation of adhesion molecules expression in inflammation may be dependent on the source of NO, the cells involved and type of stimulus used to induce inflammation.

During severe injury, infection, or ischemia and reperfusion damage, spillover of chemoactivators in the systemic circulation results in cellular activation, leading to the release of injurious agents which damage host tissues. These inflammatory mediators can alter the functional integrity of the vascular system which may be due to the upregulation of expression of cell adhesion molecules. Thus various strategies, such as monoclonal antibodies against adhesion molecules, soluble receptors, soluble counterreceptors, peptides derived from adhesion molecules to prevent receptor-ligand interactions, and antisense oligonucleotides have been employed to inhibit the cell adhesion molecules (Weiser *et al.*, 1997). Flavonoids, glucorticoid, bensothiophene- carboxamide and vitamin A have been shown to inhibit cytokine-or irradiation-induced cell adhesion molecule expression (Gerritsen *et al.*, 1995; Brostjan *et al.* 1997; Cobb *et al.*, 1996; Redlich *et al.*, 1998). Here we demonstrated that vitamin C effectively blocked the expression of leukocyte adhesion molecules. These studies suggest that vitamin C may serve as a potential therapeutic tool toward radiation-induced inflammation. Further studies are needed to clarify how this modulation occurs and to what extent it occurs *in vivo*.

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