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Effects of Rutin on Adhesion Molecules Expression and NO Production Induced by γ-irradiation in Human Endothelial cells

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Abstract – Inflammation is a frequent radiation-induced 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 bioflavonoid rutin inhibits γIR induced expression of ICAM-1, VCAM-1, and E-selectin on HUVEC in a dose- and time dependent manner. Rutin also inhibited γIR induced production of NO. These data suggest that rutin has therapeutic potential for the treatment of various inflammatory disorder associated with an increase of endothelial leukocyte adhesion molecules.

Key words ☐ Rutin, γ-irradiation, Endothelial cells, ICAM-1, VCAM-1, E-selectin, NO

The flavonoids exhibit a wide variety of biological activities, including antiviral, antibacterial, anti-inflammatory, anticarcinogenic, and antioxidant actions (Hertog *et al*, 1993; Formica *et al.* 1995). Among them, rutin, a non-toxic flavonoid glycoside with P vitamin activity, is able to suppress various free radical-mediated processes such as *in vitro* lipid peroxidation (Kozlov *et al.* 1994), the mutagenic effect of asbestos fibers on human lymphocytes (Korkina *et al.* 1992), and the overproduction of free radicals in iron-overloaded rats (Afana'ev *et al.* 1995). Recently, rutin has been shown to have the inhibitiory effects on lung inflammation and interstitial fibrosis in rats induced by bleomycine (Afanas'ev *et al.* 2001).

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 such as 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 molecule has been known to be elevated in both a temporal and spatial association with inflammatory cell infiltrates (Carlos *et al*, 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, such as 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).

Since rutin has anti-inflammatory activity and expression of adhesion molecules play an important role in inflammation, we determined whether rutin 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 rutin inhibits the upregulation of γIR-induced adhesion protein expression. We also observed that rutin blocked the production of NO induced by γIR.

MATERIALS AND METHODS

Reagents

Rutin was purchased from Sigma Chemical Co. (St Louis,

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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

HUVEC were purchased from Clonetics (San Diego, CA) and were grown in EGM-2 meidium (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 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

Rutin and γIR at concentrations presented herein were shown to be non-toxic by the following procedure. Viability of HUVEC treated with rutin and/or γIR was determined using MTT assay. Irradiated HUVEC were cultured in gelatin coated 96-well microplate (Costar Products, Cambridge, MA) until confluent. Cells were treated with or without rutin in quadruplicate for indicated time and subsequently 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) was added for 4 h. Also, 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, HUVEC were seeded at a concentration of 2×10^4 cells/well in 96-well, flat bottom, gelatin-coated plates (Nalge Nunc International, IL). The cells were incubated without or with various doses of rutin 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 phosphatase 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). The absorbance values of isotype matched control antibody were taken as blank and subtracted from the experimental values.

Nitrite determination

Irradiated HUVEC were treated with various doses of rutin 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