Ferulic Acid Attenuates Adhesion Molecule Expression in Gamma-Radiated Human Umbilical Vascular Endothelial Cells

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Radiation induces an important inflammatory response in the irradiated organs, characterized by leukocyte infiltration and vascular changes. Since adhesion molecules play an important role in facilitating the immune response at the inflammation sites, interfering with the expression of these molecules may be an important therapeutic target of radiation-induced inflammation. Many adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) have been identified in radiation. Ferulic acid (FA), an effective radioprotector during radiotherapy, is widely used in endothelium protection. The present study examined the effect of FA on the induction of adhesion molecules by gamma-radiation and the mechanisms of its effect in gamma-irradiated human umbilical vein endothelial cells (HUVECs). HUVECs were pretreated for 18 h with FA and then exposed to 10 Gy radiation. The result of cell adhesion assay showed FA inhibited radiation-induced U937 adhesion to HUVECs. FA prevented induction of ICAM-1 and VCAM-1 expression in a concentration-dependent manner after stimulation with radiation at the level of mRNA and protein. Inhibitors of the extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPKs) pathways were used to determine which pathway was involved in FA action; the result showed that the inhibitory effect of FA on adhesion molecule expression was mediated by the blockade of JNK. FA appears to be a potential therapeutic agent for treating various inflammatory disorders including radiation-induced inflammation.

Keywords: ferulic acid; radiation; intercellular cell adhesion molecule 1; vascular cell adhesion molecule 1

Radiation induces an important inflammatory response in irradiated organs, characterized by leukocyte infiltration and vascular changes. Adhesion molecules on the endothelial and non-vascular cells mediate the cell to cell interaction and play an important role in facilitating the immune response at the inflammation sites. Many adhesion molecules such as intercellular cell adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) have been identified in radiation. Ferulic acid (FA), an effective radioprotector during radiotherapy, is widely used in endothelium protection. The present study examined the effect of FA on the induction of adhesion molecules by gamma-radiation and the mechanisms of its effect in gamma-irradiated human umbilical vein endothelial cells (HUVECs). HUVECs were pretreated for 18 h with FA and then exposed to 10 Gy radiation. The result of cell adhesion assay showed FA inhibited radiation-induced U937 adhesion to HUVECs. FA prevented induction of ICAM-1 and VCAM-1 expression in a concentration-dependent manner after stimulation with radiation at the level of mRNA and protein. Inhibitors of the extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPKs) pathways were used to determine which pathway was involved in FA action; the result showed that the inhibitory effect of FA on adhesion molecule expression was mediated by the blockade of JNK. FA appears to be a potential therapeutic agent for treating various inflammatory disorders including radiation-induced inflammation.

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Reagents and Materials

FA (purity >99%) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SP600125 (a JNK inhibitor), PD98059 (an ERK inhibitor), and SB203580 (a p38 inhibitor) were purchased from Calbiochem (La Jolla, CA, U.S.A.). 3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma (Missouri, U.S.A.). Antibodies against ICAM-1, VCAM-1, JNKs, phospho-JNK (p-JNKs), p38, phospho-p38, ERK1/2, phospho-ERK1/2, and actin were purchased from Santa Cruz Biotechnology. The reverse transcription system

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and the polymerase chain reaction (PCR) system were purchased from Takara (Dalian, China). The enzyme-linked immunosorbent assay (ELISA) system and immunohistochemistry system were purchased from Boster (Wuhan, China).

**Cell Cultures and Radiation** HUVECs were obtained from Cascade Biologics (Portland, Oregon) as cryopreserved primary cultures, and grown in culture flasks in endothelial cell growth medium M200 (Cascade Biologics) supplemented with 2% LSGS (low serum growth supplement; Cascade Biologics) according to the manufacturer’s instructions. The growth medium was changed every other day until cells reached confluence. Just before radiation, the medium of confluent cells was changed and replaced with new medium. The cells were then uniformly irradiated at room temperature with 10 Gy of $^{60}$Co source (dose rate of 1.64 Gy/min), then cultured for indicated times. For each dose, control cells were simultaneously exposed to sham radiation.

**Assessment of Cell Viability** For evaluation of cytotoxicity, cells were seeded at 5000 cells/well into 96-well culture plates and grown for 24 h. Confluent HUVECs were preincubated for 18 h with the indicated concentrations of FA. The cells were then exposed to gamma radiation and cultured for 30 h, and then 20 μl of MTT was added to each well and further incubated for 4 h to allow the conversion of MTT into formazan crystals. After washing with phosphate-buffered saline (PBS), the cells were lysed with dimethyl sulfoxide (DMSO), and the absorbance read at 570 with a VictorI 1420 Multilabel Counter (Wallac, Turku, Finland).

**Cell Adhesion Assay** The methodology used for the adhesion assays is described elsewhere. Briefly, HUVECs (1×10⁵/ml), were distributed into 96-well plates and were allowed to reach confluence, then the growth medium was supplemented with FA at the indicated concentrations for 18 h, and cells were exposed to the $^{60}$Co source (10 Gy). 1×10⁶/ml U937 cells were added to each HUVEC-containing well and incubation continued for 1 h. Non-adherent cells were removed by two gentle washes with PBS, and the number of bound U937 cells was determined by MTT assay.

**Determination of Cell Surface Expression of ICAM-1 and VCAM-1 by ELISA** The ICAM-1 and VCAM-1 secretions in the medium of cell culture as described in the cell adhesion assay were analyzed by an immunoassay kit (Boster, Wuhan, China). Briefly, HUVECs (1×10⁵/ml), were distributed into 96-well plates and were allowed to reach confluence, then the growth medium was supplemented with FA at the indicated concentrations for 18 h, and cells were exposed to the $^{60}$Co source (10 Gy), and then further cultured for 6 h. A 0.1 ml volume of culture medium was incubated in the human anti-ICAM-1 or anti-VCAM-1 coated wells. The cells were treated with biotinylated anti-ICAM-1 or anti-VCAM-1, and then with streptavidin–peroxidase solution (Pierce), the human anti-ICAM-1 or anti-VCAM-1 coated wells. The fractionated proteins were electrophoretically transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington Heights) and probed with the appropriate antibodies. The blots were developed using an enhanced chemoluminescence kit (Amersham).

**Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis** At the indicated time, the cells were collected and total RNA was extracted using Trizol (Invitrogen, Carlsbad, U.S.A.) according to the manufacturer’s instructions. For RT-PCR analysis, 1 μg total RNA was reverse-transcribed using RT-PCR kits (Promega, Madison, WI, U.S.A.). PCR was used to amplify target cDNA with the following conditions: 28 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s. The PCR products were analyzed using standard agarose gel electrophoresis. Semiquantitative PCR was performed with oligonucleotides: 5′-CATG-GACCATCTACAGTTCCGG-3′ (sense) and 5′-GCTGCTACACAGTGTGATGCAA-3′ (antisense) for the ICA-M-1; 5′-CCCCCTGACCGGCTGGAGATT-3′ (sense) and 5′-CTGGGGGCAACATTGACATAAAGTG-3′ (antisense) for the ICAM-1; 5′-TCATTGACCTCAACTACATG-3′ (sense) and 5′-CAAAAGTTGTCATGGATGACC-3′ (antisense) for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Nuclear Extraction and Electrophoretic Mobility Shift Assay (EMSA)** Nuclear extracts from HUVECs were prepared using a nuclear and cytoplasmic extraction kit (Pierce, Rockford, U.S.A.). The EMSAs were carried out using a lightshift chemiluminescent gel shift kit (Pierce) according to the manufacturer's instructions. Briefly, oligonucleotides 5′-CGGCTTGATGAGTCAGCCGGAA-3′ (sense) and 5′-GCTGCTACACAGTGTGATGCAA-3′ (antisense) for the ICAM-1; 5′-CTGGGGGCAACATTGACATAAAGTG-3′ (antisense) for the VCAM-1; 5′-TCATTGACCTCAACTACATG-3′ (sense) and 5′-CAAAAGTTGTCATGGATGACC-3′ (antisense) for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

**Immunohistochemistry** The cell surface expression of adhesion molecules on endothelial monolayers was quantified using immunohistochemistry by modification of the methods described previously. Briefly, HUVECs (1×10⁵/ml), were distributed on poly-1-lysine-coated microscope glass slides and were allowed to reach confluence, then the growth medium was supplemented with FA at the indicated concentrations for 18 h, cells were exposed to the $^{60}$Co source (10 Gy), and then further cultured for 6 h. Slides were then rinsed with PBS and treated with 0.6% hydrogen peroxide in methanol to eliminate endogenous peroxidase. Thereafter, the cells were incubated with normal rabbit serum to block non-specific binding, then incubated with a polyclonal goat anti-human ICAM-1, VCAM-1 antibody. After rinses with PBS, the cells were incubated with a biotin-conjugated rabbit anti-goat immunoglobulin G, then incubated with peroxidase-conjugated streptavidin. After a final rinse, slides were stained with 3,3′-diaminobenzidine, counterstained with hematoxylin and studied using a Olympus microscope (BX40, Japan) with a 40×objective. Immunostaining was evaluated using a semi-quantitative histological score (Motic Images Advanced 3.2, Xiamen, China) which takes into account the distribution and the intensity of staining.
same sequence was added to the reaction mixture prior to the addition of the biotin-labeled oligonucleotide.

**Statistical Analysis** The data are presented as mean±S.E., and statistical comparisons between groups were performed using one-way ANOVA followed by the Student’s t test.

**RESULTS**

**FA Inhibited Radiation-Induced Monocyte Adhesion to Endothelial Cells** To examine whether FA modulates radiation-induced monocyte adhesion to endothelial cells, we examined the influence of FA on radiation-stimulated adhesion of U937 cells to HUVECs. HUVECs were pretreated without or with various concentrations of FA for 18 h and then exposed to 10 Gy of radiation. As shown in Fig. 1A, exposure to radiation resulted in an almost 2 fold increase in adhesion of U937 monocytic cells compared with adhesion of U937 cells to unstimulated HUVECs. This increase in HUVEC adhesiveness was dose-dependently reduced by preincubation of the cells for 18 h with increasing concentrations of FA, while FA had no obvious effect on non-radiated control HUVECs. As shown in Fig. 1B, pretreatment with 20 μM FA decreased radiation-induced adherence of HUVECs to U937 cells at various detected times. To determine radiation dose response on the effects of FA, HUVECs were pretreated with 20 μM FA for 18 h and then exposed to various detected radiation doses, as shown in Fig. 1C, adherence of HUVECs to U937 cells was increased in a radiation dose dependent manner, while a small decline was observed at 20 Gy of radiation, and pretreatment with 20 μM FA decreased radiation-induced adherence of HUVECs to U937 cells at various radiation doses. As shown in Fig. 2, the time of incubation and concentration of FA used in these experiments had no effect on the viability as determined by MTT assay.

**Effect of FA on Protein Expression of Adhesion Molecules** The adhesion molecules VCAM-1 and ICAM-1 played important roles in monocyte adhesion to endothelial cells; we examined protein expression of these molecules using ELISA, immunohistochemistry, and Western blot. The level of soluble adhesion molecules in supernatant was detected by ELISA, immunohistochemistry, and Western blot. The adhesion was measured as described in Materials and Methods. The results are expressed as the mean±S.E.M. of three independent experiments performed in triplicate. *Significantly different from control cells not treated with radiation or FA (∗p<0.05; ∗∗p<0.01).

**Effect of FA on mRNA Induction of Adhesion Molecules** The experiments described above demonstrated that FA markedly inhibited ICAM-1 and VCAM-1 expression in HUVECs stimulated with radiation. It was possible that FA inhibited ICAM-1 expression by modulating the levels of this adhesion molecule’s mRNA. To address this possibility, total cellular RNAs were isolated from HUVECs and analyzed by
RT-PCR using specific primers to human ICAM-1 and VCAM-1. HUVECs were pretreated with various concentrations of FA for 18 h and then exposed to 10 Gy of radiation. As shown in Fig. 6, treatment of HUVECs with FA decreased markedly the induction of ICAM-1 mRNA in a concentration-dependent manner. A similar dose-dependent decrease was seen for VCAM-1 mRNA. The level of inhibition of mRNA appeared comparable with the level of inhibition of surface expression.

Effect of FA on Radiation-Induced MAPK Signaling Pathways Since it is well understood that adhesion molecule expression is regulated by the activation of intracellular signaling proteins such as ERKs, p38s and JNKs, and these MAPK pathways are also induced by ionizing radiation, this study examined whether or not the activation of these signaling proteins was involved in the regulation of radiation-induced adhesion molecule expression. In the absence of any cytotoxicity, pretreatment with SP600125 (an inhibitor of JNK) markedly inhibited the radiation induced ICAM-1 expression, while pretreatments with either PD98059 (an ERK inhibitor) or SB203580 (a p38 inhibitor), respectively, had little or no effect on radiation-induced ICAM-1 expression (Fig. 7). The following experiments with FA were performed to examine which signaling proteins were involved in the regulation of FA inhibition of adhesion molecule expression; HUVECs were pretreated without or with various concentrations of FA for 18 h and then exposed to 10 Gy of radiation, the protein levels of p-ERK, p-JNK, p-P38 were all in-
increased in 10 Gy radiated cells, while, the total expressions were unchanged (Fig. 8). This suggested three MAPK signal pathways were all activated by radiation. FA treatment reduced the radiation mediated phosphorylation of JNK, while it did not change the level of p-ERK, and p-P38 (Fig. 8), suggesting that the inhibitory effect of FA on adhesion molecule expression is mediated by the blockade of JNK.

Effect of FA on Radiation-Induced Activation of AP-1

The AP-1 transcription factor is known to be an important mediator of adhesion molecule expression and previous results show AP-1 activation is induced by radiation. After radiation exposure, the AP-1 binding activity was quantitatively higher in the radiation exposed cells. The cells were treated with various concentrations of FA for 18 h before stimulation with radiation. The data showed that treatment with FA resulted in a decrease of radiation-mediated AP-1 activation (Fig. 9). This inhibitory effect of FA on inducible AP-1 activation was observed at a concentration range similar to that observed in inhibiting the expression of mRNA and protein of induced adhesion molecules. At 20 μM of FA, radiation-induced AP-1 activation was inhibited by 90%, as determined by densitometry scanning (data not shown). The specificity of the protein-DNA complex for the AP-1 sequence was demonstrated by competition with excess unlabeled AP-1 oligonucleotide.

DISCUSSION

Because radiation-induced vascular injury precedes the tis-
The activation of multiple MAPK pathways.

Some of the signaling radiation and a variety of other toxic stresses induces cyte adhesion.
molecule expression is of functional importance for mono-
diation at the level of mRNA and protein. In corroboration of
inflammatory responses. The present study shows that FA
has a variety of therapeutic effects, which suggests that this
compound may serve as an immunomodulator which affects
the course of inflammation. Adhesion molecules have been
known to play an important role in the regulation of cellular
inflammatory responses. The present study shows that FA
prevented induction of ICAM-1 and VCAM-1 expression in
a concentration-dependent manner after stimulation with ra-
diation at the level of mRNA and protein. In corroboration of
these results, adhesion of monocytes to endothelial cells was
markedly inhibited, indicating that the reduction of adhesion
molecule expression is of functional importance for mono-
cyte adhesion.

Based on currently available data, exposure of cells to ion-
izing radiation and a variety of other toxic stresses induces
activation of multiple MAPK pathways. Some of the sig-
naling pathways activated following radiation exposure are
ERK pathways. Other MAPK pathways activated by radia-
tion include those downstream of death receptors and procas-
pases, and DNA-damage signals, including the JNK and p38
MAPK pathways. These intracellular signal transduction
pathways are also known to regulate ICAM-1 expression in
various model systems, and activation of MAP kinases
(p38s, ERKs and JNKs) is known to promote adhesion mole-
cule expression. In order to gather further information on
the effect of FA on the radiation pathway, this study exam-
ined whether or not FA inhibited the activation of MAP kini-
ses that were among major kinases implicated in ICAM-1
signaling. In the present study, inhibitors of the ERK path-
way (PD98059), the JNK pathway (SP600125), and the p38
pathway (SB203580) were used to investigate which pathway
was involved in FA action; the result showed that the in-
hibitory effect of FA on adhesion molecule expression was
mediated by the blockade of JNK. These results are consist-
tent with our previous observation that JNK was significantly
activated by radiation, whereas the p38 and ERK pathways
did not appear to be activated. More importantly, we found
that radiation induced JNK activation was specifically inhib-
ited by FA. Thus the specific inhibitory effect of FA on JNK
activity observed in this study suggests that FA regulates AP-
1 activation by modulating JNK activity. Although FA inhibi-
tion of the JNK pathway was demonstrated, the exact target
of FA action remains to be identified. Further studies are
needed to define the target molecules of FA that transmit sig-
als from radiation to JNK activation in HUVECs.

In summary, the results of the present study demonstrate that
FA is capable of inhibiting the expression of adhesion
molecules in HUVECs, demonstrating the pharmacological
activity of FA in vascular endothelial cells. These data might
account, at least in part, for the anti-inflammatory activities
of FA. Therefore, FA appears to be a potential therapeutic
agent for treating various inflammatory disorders including
radiation induced inflammation.

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