Effect of IS-741 on Cell Adhesion between Human Umbilical Vein Endothelial Cells and HL-60 Cells

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The effect of IS-741 (N-(2-ethylsulfonylamino)-5-trifluoromethyl-3-pyridyl) cyclohexanecarboxamide monohydrate) on a model for pancreatitis has been previously reported. Recent patho-histological observations of remedial tests using rats found that the IS-741 administered group showed a low degree of tissue infiltration by inflammatory cells (polymorphonuclear leukocytes). We therefore examined cell adhesion, which is the first step in tissue infiltration by activated neutrophils, and investigated the effect of IS-741 on cell adhesion between human umbilical vein endothelial cells (HUVEC) and human promyelo-leukemia cell line (HL-60) cells during lipopolysaccharide stimulation in vitro. IS-741 significantly inhibited the adhesion of HL-60 cells to HUVEC. Further investigation of IS-741 on individual cells revealed that IS-741 mainly affected HL-60 cells. Investigation of the inhibitory effect of IS-741 at the molecular level (targeting adhesion molecules) also revealed that IS-741 had no effect on the appearance of endothelial leukocyte adhesion molecule-1 (ELAM-1), intercellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) on HUVEC, which supports the theory that IS-741 is mainly effective on HL-60 cells, even at the molecular level. However, the inhibition of adhesion was noticed in experiments in which an anti-ICAM-1 or anti-VCAM-1 antibody was added to the adhesion test system. Therefore, IS-741 is likely to affect adhesion molecules which belong to the β1 or β2 integrin family.

Key words IS-741; cell adhesion; endothelial cell; HL-60 cell; lipopolysaccharide

Generally, acute pancreatitis is believed to be a pathologic condition in which trypsinogen is activated in pancreatic tissue by a number of factors; elastase, phospholipase and trypsin are then activated for autolysis in the pancreas. Thus, at present, the most important internal medical treatment for acute pancreatitis is anti-enzyme therapy with the use of aprotinin, gabexate mesilate, nafamostat mesilate, or urinastatin.12 These drugs act by a mechanism that inhibits digestive enzymes, whereas IS-74113 does not have such inhibitory activity.

IS-741 shows marked effects in acute pancreatitis models by improving pancreatic hemorrhage, necrosis and survival rates in model animals. Its mechanism of action has not yet been elucidated. However, its effect is thought to result from reduced numbers of neutrophils adhering to vascular walls and to the reduction of inter lobular lymphocyte infiltration, determined histopathologically, which is effective against vascular injury and damage. These actions of cellular adhesion and infiltration characterize the therapeutic effects of IS-741.

On the other hand, it is known that accumulated neutrophils cause various diseases, including acute pancreatitis.3-9 Neutrophils adhere to vascular walls through various adhesive molecules. Adhered and activated neutrophils produce and release leukotriene B4 (LTB4), elastase, platelet activating factor (PAF), active oxygen, and other substances. Tissue collapse is induced by the excessive release of these substances. It is also assumed that chemical mediators such as LTB4 and PAF released at that time induce the further accumulation of neutrophils, a vicious cycle that worsens pancreatitis.

In this study, we focused on the fact that IS-741 inhibits adhesion and infiltration of neutrophils in vivo, and attempted to verify cellular adhesion inhibitory effects as an action mechanism of this drug in vitro. Here, we report the results of the adhesion study using a human promyelocytic leukemic cell line (HL-60) and human umbilical vein endothelial cells (HUVEC).

MATERIALS AND METHODS

Agent, rTNFα and Antibodies The IS-741 was a sodium salt of N-(2-ethylsulfonylamino)-5-trifluoromethyl-3-pyridyl] cyclohexanecarboxamide monohydrate. IS-741 was prepared as a 10 molar solution just before use. Cyclosporin A was obtained from Novartis Pharmaceuticals. Human recombinant tumor necrosis factor α (TNF) (sp. Act. of 2.5×10^7 U/mg) was raised in our laboratory.10 Anti-intercellular adhesion molecule-1 (ICAM-1) (LB-2) and anti-endothelial leukocyte adhesion molecule-1 (ELAM-1) (H18/7) antibodies were obtained from Becton Dickinson. Anti-vascular cell adhesion molecule-1 (VCAM-1) (1G11) antibody was obtained from Cosmo Bio Co., Ltd.

Cell Isolation and Cultures Endothelial cells were harvested from human umbilical veins by collagenase perfusion by modifying the method of Jaffe et al.11 Cells were maintained in MCDB107 (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan) supplemented with endothelial cell growth supplement (100 μg/ml; Collaborative Research Inc.), heparin (100 μg/ml; Sigma), penicillin (100 units/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS) (Gibco). HUVEC were grown on 35 mm gelatin precoated tissue culture plates (Corning) and were used up to passage five.

HL-60 (a human promyelocytic leukemia line; ATCC, CCL-240) was grown in RPMI 1640 (Sigma) supplemented with 15% FBS (Gibco), penicillin (100 units/ml) and streptomycin (100 μg/ml).

Assay for Cell Adhesion to HUVEC Pre-coated (0.5% gelatin) 96-well culture plates (Corning) were used to assay cell adhesion. HUVEC were plated and grown to confluence. HUVEC were washed once and incubated with 50 ng/ml lipopolysaccharide (LPS) (Gibco) for 4h at 37°C. HL-60

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cells were added (2×10^4 cells/well) to the LPS stimulated HUVEC and allowed to attach for 30 min at 37°C. Non-adherent cells were then removed and the cells were washed three times with cell culture medium (MCDB107). Adherent cells were fixed in 3.7% phosphate buffered formalin. The number of HL-60 cells that adhered to HUVEC were counted under a phase-contrast microscope (Nikon) using a digital photograph system (Sony), which measured an area of 0.16 mm^2/field.

**Assays for ICAM-1, VCAM-1 and ELAM-1 Expression on HUVEC** Confluent cultures of HUVEC were stimulated with LPS (100 ng/ml). HUVEC were fixed with 3.7% buffered formalin, then washed with 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (BSA buffer) four times. Fc receptors were blocked by incubation with horse serum (diluted 1:50 in BSA buffer) for 1 h at 37°C. The cells were then washed and incubated with 1 μg/ml anti-ICAM-1 (LB-2), 400 ng/ml anti-VCAM-1 (1G11) or 1 μg/ml anti-ELAM-1 (H18/7) antibody for 1 h at 37°C. Then the cells were washed three times and incubated with biotinylated horse anti-mouse IgG with BSA buffer for 1 h, followed by another washing and incubation with avidin-biotinylated peroxidase (for ICAM-1 and ELAM-1 expression) or alkaline phosphatase-streptavidin (for VCAM-1 expression). After the cells were washed three times, the substrates for peroxidase, TMB (3,3',5,5'-tetramethylbenzidine), and for alkaline phosphatase, p-nitrophenolphosphate, were added. The reaction was stopped (by 4 N HSO_4 or 2 M NaOH, respectively) and absorbance was measured at 450 and 405 nm, respectively.

**Statistical Analysis** The significance of any differences was determined using Williams’ test, Student’s t-test or Aspin-Welch’s r-test, and p values below 0.05 were considered statistically significant.

**RESULTS**

**Effect of IS-741 on Cell Adhesion between HUVEC and HL-60 Cells** As seen in Fig. 1, LPS-stimulation of HUVEC for 4 h resulted in a marked increase of HL-60 cell adhesion (100% stimulated vs. 5% unstimulated). IS-741 was preincubated 1 h before contact cultivation of the cells. Under this experimental condition, the suppression rates of IS-741 against LPS-stimulate were 56% and 28% with the treatments of 1 and 0.1 μM of IS-741, respectively. It was also examined whether TNF stimulated increases in HL-60 cell adhesion to HUVEC (Fig. 2). The adhesion of HL-60 cells increased following 4 h of incubation of TNF (100% stimulated vs. 2% unstimulated). IS-741 significantly suppressed cell adhesion between HUVEC and HL-60 cells stimulated by TNF. The suppression rate was 38% with the treatment of 1 μM of IS-741.

**Direct Inhibitory Effect of IS-741 on Cell Adhesion**

The A group, which consisted of HUVEC and HL-60 cells treated with LPS, showed strong adhesion activity even in the absence of LPS at the time of contact cultivation (Fig. 3). The B group, which consisted of HUVEC and HL-60 cells treated with LPS and IS-741, showed strong suppression of adhesion even in the absence of IS-741 at the time of contact cultivation. IS-741 was preincubated 1 h before contact cultivation of the cells. If the adhesion rate of the A group was 100%, that of the B group was 36%, which was statistically significant. On the other hand, in the C group in which HUVEC and HL-60 cells were stimulated by LPS, IS-741 was only present at the time of contact cultivation. If IS-741 had direct suppression activity on adhesion molecules, the degree of adhesion should be less than that of the A group. However, the results show the same degree of adhesion as the A group, Fig. 3. This finding suggests that IS-741 has no effect of direct inhibition on the adhesion of both types of activated cells.

**Influence of IS-741 on the Activation of HUVEC**

As seen in Fig. 4, the adhesion rate of the B group, which underwent LPS-stimulation for 3 h before IS-741 was added and
Fig. 3. The Direct Inhibitory Effect of IS-741 on the Adhesion of HL-60 Cells to HUVEC

HUVEC and HL-60 cells were stimulated with LPS for 4 h and 30 min, respectively, but LPS was removed from contact cultivation by culture media exchange. In the B group, IS-741 (1 μM) was added to HUVEC 3 h following LPS-stimulation. The C group was treated with IS-741 only in contact cultivation. Each value represents the mean±S.D. of five fields. Significantly different from the LPS stimulated group (A), *p<0.001.

Fig. 4. Effect of IS-741 Pretreatment to HUVEC on the Adhesion of HL-60 Cells Associated with LPS-Stimulation

HUVEC and HL-60 cells were stimulated with LPS for 4 h and 30 min, respectively, but LPS was removed from contact cultivation by culture media exchange. In the B group, IS-741 (1 μM) was added to HUVEC 3 h following LPS-stimulation. Each value represents the mean±S.D. of six fields. Significantly different from the LPS stimulated group (A), *p<0.001.

treated for 1 h, was 92% of that of the A group. The suppression rate of IS-741 on cell adhesion by LPS-stimulation was 8%, indicating that slight suppression occurred, although it was not significant. On the other hand, when HUVEC were not stimulated by LPS (the C group), although HL-60 cells were stimulated by LPS, the adhesion rate was only 4%. This result indicates that IS-741 has a weak effect on HUVEC alone.

Influence of IS-741 on the Activation of HL-60 Cells

As seen in Fig. 5, the C group, in which only HUVEC were treated by LPS and HL-60 cells were not treated, showed an adhesion rate of 50% compared with the A group (100%). This result and Fig. 4 show that the activation of HUVEC is indispensable for HL-60 cell adhesion. Also, this result showed that HL-60 cell adhesion to HUVEC was increased significantly (50%) by LPS-stimulation of HL-60 cells. On the other hand, IS-741 almost completely suppressed (93%) the increased adhesion of HL-60 cells induced by LPS-stimulation.

Fig. 5. Effect of IS-741 Pretreatment of HL-60 Cells on the Adhesion to HUVEC Associated with LPS-Stimulation

HUVEC and HL-60 cells were stimulated with LPS for 4 h and 30 min, respectively, but LPS was removed from contact cultivation by culture media exchange. In the B group, IS-741 (1 μM) was co-treated to HL-60 cells for LPS-stimulation. Each value represents the mean±S.D. of six fields. Significantly different from the LPS stimulated group (A), *p<0.001.

Influence of IS-741 on the Adhesion Molecules of HUVEC

The quantity of adhesion molecules appearing on HUVEC by LPS-stimulation was examined. On the surface of HUVEC, ELAM-1 was scarcely expressed constitutively and its absorbance was measured as 0.044, but the degree of expression increased to 0.638 with LPS-stimulation. The IS-741 treated group showed an absorbance of 0.555, and there was no influence on the expression of ELAM-1 induced on HUVEC by LPS-stimulation (Fig. 6A). ICAM-1 was expressed constitutively as low as 0.024. The degree of expression increased to 0.148 with LPS-stimulation. The IS-741 treated group showed an absorbance of 0.134, and there was no influence on the expression of ICAM-1 induced on HUVEC by LPS-stimulation (Fig. 6B). VCAM-1 was scarcely expressed constitutively, and its value was as low as 0.008, but the degree of expression increased to 0.204 with LPS-stimulation. The IS-741 treated group showed an absorbance of 0.180, and it slightly suppressed the expression of VCAM-1 induced on HUVEC by LPS-stimulation, but it was not highly active (Fig. 6C). Against these results, cyclosporin A, which was used as the positive control, significantly suppressed the expression of all adhesion molecules examined.

Influence of Anti-adhesion Molecule Antibodies

Anti-ICAM-1 and anti-VCAM-1 antibodies suppressed cell adhesion to the same degree as the IS-741 treated group. As for anti-ELAM-1 antibody, it did not show any suppression of adhesion (Fig. 7). When anti-ICAM-1 and anti-VCAM-1 antibodies were mixed, the degree of suppression increased slightly more than individual treatment by anti-ICAM-1 or anti-VCAM-1 antibody. With anti-ELAM-1 antibody alone, the suppression of adhesion was not shown, and even when anti-ELAM-1 antibody was combined with either anti-ICAM-
DISCUSSION

IS-741 significantly inhibited cell adhesion between HUVEC and HL-60 cells stimulated by LPS. In the current adhesion study, the inhibition rate, when IS-741 was added 3 h after LPS-stimulation of HUVEC at concentrations of 1 and 0.1 μM, were 56% and 28%, respectively (Fig. 1). When the cell stimulant was changed to TNF, the degree of suppression was weaker but the results were almost the same (Fig. 2). Also, both cells were stimulated individually by LPS. LPS was removed by washing with culture medium, and the cell adhesion experiments were carried out in the absence of LPS. With this system, the same degree of cell adhesion as in the presence of LPS was observed. Consequently, in order to investigate the effect of IS-741 on the cell adhesion, various experiments were conducted. First of all, the possibility of direct suppression by IS-741 was investigated. The results indicated that IS-741 did not directly suppress the adhesion of both activated cells (Fig. 3). Then, using a system in which each cell was separately treated with IS-741, the adhesion inhibitory effect of IS-741 (as shown in Fig. 1) was analyzed to determine whether it affected either HUVEC or HL-60 cells. IS-741 inhibited the adhesion activity of HUVEC induced by LPS-stimulation by 8% (Fig. 4). Conversely, IS-741 inhibited the adhesion activity of HL-60 cells induced by LPS-stimulation by 93%, which confirmed that the inhibition activity was clearly more distinct on the HL-60 cell side (Fig. 5). Moreover, the effect of IS-741 was investigated for constitutive adhesion of HL-60 cells. It was suggested that the constitutive adherence activity of HL-60 cells was unaffected by IS-741 treatment (data not shown). It is evident that the adhesion between cells is induced by cell adhesion molecules. How IS-741 affects the expression of cell adhesion molecules requires further analysis at the molecular level. Therefore, it was undertaken to determine whether IS-741 inhibited the expression of ELAM-1, ICAM-1 and VCAM-1 molecules induced by LPS-stimulation on HUVEC. IS-741 did not show any inhibitory effect on ELAM-1, ICAM-1 or VCAM-1 (Fig. 6A, B and C). Among the substances known to show adhesion inhibition are steroids and cyclosporin A (immunosuppressant), and these substances reveal the activity by suppressing the appearance of adhesion molecules on blood vessel endothelial cells.12,13) Because IS-741 does not induce such activity, however, its adhesion inhibitory activity is probably due to a mechanism...
other than those of the substances above. To elucidate such a new mechanism, we analyzed IS-741’s target molecules in HL-60 cells. By adding anti-ICAM-1 or anti-VCAM-1 antibody to the adhesion test system, cell adhesion was inhibited (Fig. 7), which indicated the intensified participation of adhesion molecules of the immunoglobulin family in the cell adhesion system we observed. Therefore we tried an adhesion blocking test using anti-VLA-4 antibody and/or anti-CD18 antibody for HL-60 cells. As a consequence, each antibody suppressed the cell adhesion, in which the combination of both antibodies only slightly increased the suppression level as compared with that achieved by the individual treatment. In these conditions, IS-741 did not show any additional suppression of cell adhesion (data not shown). If we consider the strength of reactivity of IS-741 to HL-60 cells as the target for this drug, we must first consider the integrin family.14—17

Our conclusion based on the above results is that IS-741 significantly inhibits the adhesion of LPS-stimulated HUVEC and HL-60 cells at concentrations as low as 1 and 0.1 μM, and that such adhesion inhibition proved effective by the reaction of IS-741 mainly on the HL-60 cell side. We speculate that IS-741 targets the adhesion molecules (such as LFA-1 or Mac-1) which exist on the surface of HL-60 cells or which promptly appear, then inhibits the environment in which such molecules are activated (expression or structural change) by LPS-stimulation, resulting in the inhibitory effect.

REFERENCES