Sivelestat Sodium Hydrate Inhibits Neutrophil Migration to the Vessel Wall and Suppresses Hepatic Ischemia-Reperfusion Injury

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Sivelestat Sodium Hydrate Inhibits Neutrophil Migration to the Vessel Wall and Suppresses Hepatic Ischemia–Reperfusion Injury

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Abstract

Background Sivelestat sodium hydrate (sivelestat) is a specific neutrophil elastase inhibitor that is effective in treating acute lung injury associated with systemic inflammatory response syndrome. As such, it may be useful in treating hepatic ischemia–reperfusion injury (IRI), a condition in which neutrophils transmigrate into the interstitium, leading to release of neutrophil elastase from neutrophils and consequent damage to the affected tissue, particularly in cases of hepatic failure after liver transplantation or massive liver resection.

Aims The purpose of this study was to examine whether treatment with sivelestat inhibits neutrophil adhesion and migration to the vessel wall and suppresses hepatic IRI.

Methods Whether and, if so, the extent to which sivelestat suppresses the adhesion and migration of neutrophils and reduces liver damage in hepatic IRI was examined in a human umbilical vein endothelial cell (HUVEC) model and a rat hepatic IRI model.

Results In the HUVEC model, the extent of the adhesion and migration of neutrophils stimulated by platelet-activating factor were found to be dose-dependently inhibited by sivelestat treatment ($p < 0.05$). In the rat model, serum

liver enzyme levels were significantly lower at 12 h after reperfusion, and the number of neutrophils that had migrated to extravascular sites was significantly less in the treatment group compared to the control group ($p < 0.05$). **Conclusion** Sivelestat inhibits the adhesion and migration of neutrophils to vascular endothelium in hepatic IRI, thereby suppressing liver injury.

Keywords Ischemia–reperfusion injury · Liver failure · Neutrophil elastase inhibitor · Sivelestat sodium hydrate · Systemic inflammatory response syndrome

Introduction

Ischemia–reperfusion injury (IRI) of the liver has been demonstrated in a variety of clinical settings, such as liver transplantation and hepatic failure after massive liver resection [1]. The possible consequences of IRI include both primary severe liver dysfunction and secondary multi-organ system failure that eventually lead to mortality [2–4]. The mechanisms underlying hepatic IRI are complex but are known to involve leukocyte accumulation and activation (neutrophils, Kupffer cells, and T cells), leading to the formation of reactive oxygen species (ROS), secretion of pro-inflammatory cytokines/chemokines, complement activation, and vascular cell adhesion molecule activation [5–7]. ROS and tumor necrosis factor alpha (TNF- α) released from Kupffer cells [8, 9], complement [10], platelet-activating factor (PAF) [11], endothelin-1 [12] and superoxide are reportedly involved in IRI. Neutrophil activation has long been considered the major effector mechanism in hepatic IRI [13–15]. The rolling of neutrophils is an important prerequisite for adhesion and migration into tissues, and a two-step

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leukocyte recruitment process has been established [16]. The migration of neutrophils into the parenchyma is a prerequisite for neutrophil-mediated injury [17]. Neutrophil elastase is a serine protease found in the azurophilic granules of neutrophils. The requirement for neutrophils to migrate out of the vasculature and through the basement membrane, as well as the potent proteolytic function of neutrophil elastase, have led to the theory that neutrophil elastase might be involved in the pathogenesis of inflammatory tissue injury such as that exemplified by liver IRI. Sivelestat sodium hydrate (Elaspol, ONO-5046Na; Ono Pharmaceutical, Osaka, Japan) is a synthetic, low-molecular-weight, specific inhibitor of neutrophil elastase [18]. In several studies involving animal models, sivelestat was effective in alleviating acute lung injury (ALI) [19, 20] and liver injuries [21, 22]. However, there are few reports on the relationship between sivelestat and the kinetics of neutrophils. In this study, we used human umbilical vein endothelial cells (HUVEC) and a rat hepatic IRI model to demonstrate that sivelestat inhibits adhesion and transmigration of neutrophils to the vessel wall and suppresses hepatic IRI.

Materials and Methods

Sivelestat Sodium Hydrate

Sodium *N*-[2-[4-(2,2-dimethylpropionyloxyp)phenylsulfonylamino]-benzoyl] amino acetate tetrahydrate (ONO-5046; C₂₀H₂₁NaO₇S/H₂O; mol wt. 528.51) was provided by the Ono Pharmaceutical Company, Osaka, Japan.

Neutrophils

Human neutrophilic polymorphonuclear leukocytes were isolated from venous blood of healthy adults using standard dextran sedimentation and gradient separation on Histopaque 1077 (Sigma-Aldrich) [23]. This procedure yields a polymorphonuclear leukocyte population that is 95–98 % viable (trypan blue exclusion) and 98 % pure (acetic acid—crystal violet staining).

Endothelial Cells

HUVEC were harvested from umbilical cords by collagenase treatment as previously described [23]. The cells were plated in HuMedia-SG2 (Kurabo Inc., Japan) supplemented with fetal bovine serum 25 mL, hEGF 0.5 mL, hFGF-B 0.5 mL, insulin 0.5 mL, and antibiotics (amphotericin B). The cell cultures were incubated at 37 °C in a humidified atmosphere with 5 % CO₂ and expanded by brief trypsinization (0.25 % trypsin in phosphate-buffered saline

containing 0.02 % EDTA). Primary through third passage HUVEC were used in the experiments.

Adhesion Assay

HUVEC were grown to confluence on fibronectin (25 µg/mL) coated Falcon cell culture inserts (six wells, 3-µm diameter pores). Neutrophils collected from healthy adults labeled using a PKH2 Green Fluorescent Cell Linker Kit (Sigma-Aldrich) were stimulated with PAF (0.1 mM) or not and added to the HUVEC monolayers and co-incubated for 1 h with various concentrations (1, 10, and 50 µg/mL) of sivelestat. After 1 h, neutrophils remaining in the chamber were washed twice and counted using a BIO-REVO BZ-9000 microscope (Keyence, Osaka, Japan) in ten different high power fields.

Migration Assay

HUVEC were grown to confluence on fibronectin (25 µg/mL) coated Falcon cell culture inserts (six wells, 3-µm diameter pores). Neutrophils stimulated by PAF (0.1 mM) were added to the HUVEC monolayers (upper chamber). The upper chamber was exposed to 2 mL of HUMEDIA and rehydrated at 37 °C for 1 h in the absence or the presence (50 µg/mL) of sivelestat. Subsequently, the upper chamber was removed and the fluid in the lower chamber was collected. The neutrophils in 1 mL of fluid were counted using an Attune Acoustic Focusing Flow Cytometer (Applied Biosystems, USA).

Animals

Male Wister rats (250–300 g, Charles River Inc., Japan) were used. The animals were allowed free access to water and standard laboratory chow. They were fasted for 24 h before the surgical procedure. The present study was conducted in compliance with the Division for Animal Research Resources, University of Kanazawa. The experiments and procedures were approved by the Animal Care and Use Committee of the University of Kanazawa.

Ischemia–Reperfusion Injury (IRI) Model

The animals were randomly divided into two groups: sivelestat and control. The animals were anesthetized by inhalation of diethyl ether and injected with heparin (100 U/kg). A midline incision was made and the liver was exposed. The hepatoduodenal ligament was clamped with a hemostasis clip. After 30 min of total hepatic ischemia, the clamp was removed to initiate hepatic reperfusion. Sivelestat (30 mg/kg) was injected into the inferior vena cava 5 min before total hepatic ischemia. At the indicated times

(6, 12, and 24 h) after reperfusion, the rats were killed ($n = 8$ each) for collection of serum and liver tissues.

Biochemical Analysis

To evaluate liver injury at each time point, serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were measured using the Japan Society of Clinical Chemistry standardization matching method. All measurements were performed by SRL Inc., Japan.

Histological Analysis

Liver tissue was fixed in 10 % neutral buffered formalin, embedded in paraffin, and cut serially into 5- μ m sections. The hematoxylin and eosin (H&E) stained sections were evaluated at 400 $\times$ and 100 $\times$ magnifications.

Statistical Analysis

All results were expressed as means $\pm$ standard deviations (SD). Comparisons between the two groups were performed with Student's *t* test or the Mann–Whitney *U* test, as appropriate. A *p* value of less than 0.05 was considered statistically significant.

Results

Adhesion Activity

Neutrophils stimulated by PAF (0.1 mM) were incubated on HUVEC monolayers for 1 h at 37 °C in a humidified atmosphere of 5 % CO₂ with three concentrations (0, 10, and 100 μ g/mL) of sivelestat. Addition of 10 μ g/mL sivelestat completely prevented adhesive activity. However, addition of 100 μ g/mL sivelestat changed HUVEC and neutrophil morphologies. Therefore, neutrophils were seeded on HUVEC monolayers with various concentrations of sivelestat (0, 1, 10, and 50 μ g/mL). Addition of 1 μ g/mL sivelestat (145 ± 37 cells/field, $n = 10$) significantly inhibited adhesion to HUVEC monolayers as compared to the absence of sivelestat (369 ± 61 cells/field, $n = 10$) ($p < 0.05$). There was no significant difference between 1 μ g/mL, 10 μ g/mL and 50 μ g/mL sivelestat (Fig. 1).

Migration Activity

As shown in Fig. 2, 0.1 mM of PAF significantly increased the number of neutrophils migrating through the membrane (284 ± 38 cells/mL, $n = 6$) as compared to number of neutrophils without PAF treatment (158 ± 29 cells/mL,

$n = 6$) ($p < 0.01$). Addition of 50 μ g/mL sivelestat with PAF (0.1 mM) significantly reduced the number of neutrophils migrating through the membrane (221 ± 35 cells/mL, $n = 6$) ($p < 0.05$) (Fig. 2).

Effects of Sivelestat on Hepatic IRI

Hepatocellular injury was evaluated by measuring liver enzymes (AST, ALT, and LDH). Serum AST, ALT, and LDH levels were significantly lower in the sivelestat group than in the animals receiving normal saline solution (783 ± 371 vs. 358.2 ± 90.3 IU/L, 471 ± 195.1 vs. 165.4 ± 91.2 IU/L, $2,289 \pm 756.5$ vs. 590.8 ± 299.4 mg/dL, respectively) at 12 h after IRI ($p < 0.05$, Fig. 3). Serum levels of AST were lower in the sivelestat group than in the saline group (366.8 ± 104.4 vs. 227.4 ± 15 IU/L) at 24 h after IRI ($p < 0.05$, Fig. 3).

Histopathological Analyses of IRI Specimens

In the control group, many neutrophils had migrated into the connective tissue of Glisson's capsule at 12 h. In the sivelestat group, fewer neutrophils migrated to extravascular sites than in the control group (Fig. 4).

Discussion

Neutrophil elastase is a 30 kD neutral serine protease stored in an active form in the azurophil granules of neutrophils. Neutrophils can be stimulated to release elastase upon exposure to various cytokines and chemoattractants, including TNF α [24], interleukin-8, complement component 5a [25], lipopolysaccharide [26], and a tripeptide derived from bacterial walls (*N*-formyl-methionyl-leucyl-phenylalanine) [27]. In the physiological state, neutrophil elastase includes most components of the extracellular matrix (e.g., collagen, fibronectin, and laminin) as well as a wide range of other proteins such as cytokines, clotting factors, adhesion molecules, and components of the complement cascade [28]. With morbidity, neutrophil elastase inactivates elastic fibers, proteoglycans, collagen fibers, antithrombin III, and the α 2-plasmin inhibitor. Antithrombin III is inactivated via heparin-binding neutrophil elastase acting directly on it, thereby causing disseminated intravascular coagulation. It has been proposed that elastase-mediated degradation of the endothelial basement membrane facilitates neutrophil transit into the interstitium [29]. Once extravasated, neutrophils will adhere to the target, i.e., parenchymal cells. The migration of neutrophils into the parenchyma is a prerequisite for neutrophil-mediated injury [17]. There is general agreement on the mechanisms involved in neutrophil adhesive interactions.

Fig. 1 Neutrophil adhesion inhibitory effects of sivelestat. Neutrophils labeled with PKH2 green fluorescent cell linker kit (Sigma-Aldrich) stimulated by PAF (0.1 mM) were seeded with various concentrations (1, 10, and 50 $\mu\text{g}/\text{mL}$) of sivelestat on HUVEC monolayers. After incubation for 1 h at 37 °C in a humidified atmosphere of 5 % CO_2 in an incubator, the neutrophils adhering to HUVEC in ten different high power fields (HPFs) were measured using a BIOREVO BZ-9000 microscope (Keyence, Osaka, Japan). Data are expressed as mean $\pm$ SE from 10 HPFs

Neutrophil elastase is clearly involved in the migration of neutrophils. Within the living body, host tissues are protected from unregulated proteolysis by neutrophil elastase by antiproteases such as α_1 -proteinase inhibitor, secretory leukoprotease inhibitor, α_2 -macroglobulin, and egli [30, 31]. Nonetheless, neutrophils can resist these anti-proteases via four processes. First, neutrophils are able to create a relatively sequestered “microenvironment” or “protected space” in the subjacent area encompassing the neutrophil and the surface to which it is adherent [32]. Second, anti-proteases are sensitive to inactivation by oxidants released from activated neutrophils, which oxidize a critical methionine residue in the active site [33, 34]. Third, neutrophil elastase that is bound to elastin is relatively resistant

to inhibition by anti-proteases [35]. Finally, activated neutrophils have been shown to express neutrophil elastase on the cell surface; this elastase is active and resistant to inhibition by anti-proteases [36]. Furthermore, neutrophil elastase induces adhesion molecules such as selectins and β_2 integrin/intercellular adhesion molecule-1 (ICAM-1) or β_1 integrin/vascular adhesion molecule-1 interactions [37, 38]. Strong adhesion and transmigration processes trigger the exocytosis of gelatinase granules from neutrophils, which liberate matrix metalloproteinases [39]. Several in vivo and in vitro experiments have demonstrated that protease inhibitors exert a hepatoprotective effect against IRI, in association with suppression of the aforementioned factors [40–43]. However, few studies have focused on the

Fig. 2 Neutrophil migration inhibitory effects of sivelestat. Neutrophils stimulated by PAF (0.1 mM) were added to HUVEC monolayers (*upper chamber*). The *upper chamber* was exposed to 2 mL of HUMEDIA and rehydrated at 37 °C for 1 h in the absence or the presence (50 µg/mL) of sivelestat. One milliliter of the fluid in the *lower chamber* was examined using an Attune Acoustic Focusing Flow Cytometer (Applied Biosystems, USA). Data are expressed as mean $\pm$ SE of six wells

Fig. 3 Inhibition of hepatic enzyme release. Rats were infused with sivelestat or saline solution at the time of reperfusion. Serum levels of aspartate aminotransferase (AST) levels, serum alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were measured at the indicated time points. Values are mean $\pm$ SE, * p < 0.05, ** p < 0.01 versus saline solution

relationship between sivelestat and the kinetics of neutrophils. We reaffirmed that sivelestat reduces hepatic IRI, as reflected by the serum AST, ALT, and LDH levels being significantly lower in the sivelestat group. This assessment revealed that sivelestat suppresses both adhesion and

transmigration of neutrophils to the endothelium. It is difficult for the α 1-proteinase inhibitor (53 kD) and α 2-macroglobulin (720 kD) to gain access to the microenvironment because these are large molecules compared with neutrophil elastase (30 kD). However, sivelestat can access

Fig. 4 Representative liver histology (hematoxylin-eosin staining; magnification, $\times 400$) of ischemic (30 min) liver lobes harvested 12 h after reperfusion. Sivelestat treatment (30 mg/kg) ameliorated hepatic ischemia–reperfusion injury and suppressed transmigration of neutrophils into the connective tissue of Glisson’s capsule

the microenvironment because of its small size. We speculate that sivelestat inhibits neutrophil elastase in the microenvironment, such that adhesion and transmigration are suppressed. In fact, the histopathological analyses revealed fewer neutrophils transmigrating to the interstitium in the sivelestat group. ICAM-1 expression in hepatic IRI is also reportedly inhibited by sivelestat [44]. The adhesion and migration assays demonstrated that sivelestat significantly reduced the adhesion and migratory activities of neutrophils. In clinical research, sivelestat was administered to shorten the duration of systemic inflammatory response syndrome (SIRS) in patients undergoing video-assisted thoracoscopic surgery for esophageal cancer [45]. In the same study, postoperative peripheral white blood cell (WBC) counts were generally higher in the sivelestat-treated group than in the control group. The higher peripheral blood WBC counts in the sivelestat-treated group might reflect the effectiveness of this neutrophil elastase inhibitor in suppressing neutrophil transmigration from circulating blood to the vessels of organs, such as the lungs and liver, leading to the prevention of ALI. In our present study, we counted the number of peripheral blood WBCs in a rat model, but no significant increase was found (data not shown). However, the durations of peripheral blood WBC elevation differ between humans and rats. Sivelestat is now recognized as being clinically effective for reducing ALI associated with SIRS. In this

study, we confirmed that sivelestat suppressed adhesion and transmigration to blood vessel walls in a hepatic IRI model. We can thus reasonably speculate as to one of the mechanisms by which sivelestat may reduce hepatic IRI. We advocate that sivelestat be used prophylactically for advanced invasive surgery, such as liver transplantation and massive liver resection that can cause SIRS. Therefore, we started a clinical trial of sivelestat treatment for the prevention of SIRS in patients receiving advanced invasive surgery. On the other hand, prolonged use of sivelestat, for SIRS due to infectious diseases or sepsis, necessitates caution because there is a possibility of excessive inhibition of the normal functions of neutrophils. In conclusion, sivelestat suppresses liver injury by inhibiting the adhesion and transmigration of neutrophils to the vascular endothelium. Sivelestat has therapeutic potential for the prevention and treatment of hepatic injury due to ischemia–reperfusion.

Conflict of interest None.

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