Development of a Simple Permeability Assay Method for Snake Venom-induced Vascular Damage

Kae SATO,† Ayuki KODAMA,* Chikako KASE,† Satoshi HIRAKAWA,** and Manabu ATO***

*Department of Chemical and Biological Sciences, Faculty of Science, Japan Women’s University, Bunkyo, Tokyo 112–8681, Japan
**Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431–3192, Japan
***Department of Immunology, National Institute of Infectious Diseases, Shinjuku, Tokyo 162–8640, Japan

We have developed a novel bioassay method for the detection of snake venom based on the permeability of endothelial cell monolayers cultured in Transwell cell culture inserts. This assay relies on the proteolytic degradation of capillary basement membrane proteins, a pathophysiological event that occurs due to snakebites in vivo. Transwell permeability assays with fluorescence measurements are advantageous with regard to ethical considerations for the use of animals. The assay time was reduced from 24 h for animal tests to 2 h, and many samples could be assayed easily.

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Introduction

Snake venom contains hemolytic toxins, which attack the walls of blood vessels.1 The main component of hemolytic toxins is proteolytic enzymes. These enzymes destroy blood vessel walls through their proteolytic activity; thus, snake bites cause hemorrhage and tissue damage. Antiserum against snake venom has applications in the clinical treatment of venomous bites to neutralize the lethal and hemorrhagic activities of the venom. In Japan, these antitoxin formulations, such as those against the venom of habu (Protobothrops flavoviridis), a major venomous snake in Japan, can be provided only after receiving certification based on national standards. However, antivenom potency testing is performed only once every 3–5 years. The antihemorrhagic potency test, which is a major test for measuring antivenom activity, is an animal test using rabbits.2,3 This method is based on the intradermal injection of venom solution into a rabbit, followed by a measurement of the hemorrhagic spot area in the inner side of the skin, requiring more than 24 h. However, because of animal-welfare concerns, the World Health Organization (WHO) recommends the development of alternative methods to animal testing in the preclinical evaluation of antivenom.4

Habu snake venom has a number of protease activities.5 A serine proteinase in Habu snake venom is known to function as a blood coagulation factor.6 Additionally, a metalloproteinase in Habu snake venom damages capillary endothelial cells and degrades the extracellular matrix in the vascular wall.7,8 In a previous study, BODIPY-FL-labeled casein was used in a habu snake venom protease activity test conducted without either animals or cells.9,10 In this method, the proteolytic cleavage-mediated increase in the fluorescence quenching of BODIPY-FL-labeled casein was measured.10 This method required a shorter assay time (~1 h) than that required for the antihemorrhagic potency test performed using rabbits. However, the results obtained by the proteolytic cleavage activity test were reported to not necessarily correspond to those of the hemorrhagic-activity tests,9 which indicates that hemorrhagic activity cannot be predicted based only on proteolytic cleavage assays performed using isolated proteins. Schneider et al.11 developed a snake venom detection biosensor, also based on proteolytic cleavage assays, and similar problems were encountered.

As an alternate approach to animal tests, the analysis of damage to endothelial cells cultured in vitro may be used to predict hemorrhagic activity. For example, Diaz et al.12 reported an assay based on the apoptosis of a human umbilical vein cell line, EA.hy926, induced by a metalloproteinase in the venom of a poisonous snake, Bothrops asper. Additionally, Wang et al.13 reported an assay based on changes in the human umbilical vein endothelial cell (HUVEC) morphology and nuclear fragmentation by purified fibrinolytic enzyme (FHa) from Agkistrodon acutus venom. However, physiological phenomena related to hemorrhagic activity have not been examined.

Recently, in vitro vascular permeability assays have been performed using Transwell cell culture inserts.14 The permeability of monolayer-cultured vascular endothelial cells was measured by the following procedure. First, vascular endothelial cells were cultured in monolayers on a permeable membrane in cell culture inserts. Fluorescence tracer molecules were added to the cell culture inserts (luminal compartment) on which endothelial cells were cultured. If the endothelial cell monolayer had a hole, fluorescence tracer leaked through the membrane. The fluorescence intensity of the lower component
Based on this assay, we have reported a microfluidic-based blood and lymphatic vascular transmembrane permeability assay system. The microfluidic devices required small volumes of culture media and small quantities of cells, leading to reduced running costs, and realized perfusion cell culture, similar to the vascular flow. Using a microfluidic channel with a porous membrane, we observed an increase in vascular transmembrane permeability induced by habu snake venom within 30 min. If inhibition of the increase in vascular permeability can be experimentally verified, the assay may be an alternative to conventional hemorrhagic spot analysis. However, assays using commercially available Transwell inserts may be more useful than those using a microfluidic device for researchers studying snake venom because microfluidic devices are not readily available at present. In addition, to increase the performance of the assay, cell lines are preferable to human normal blood and lymphatic vascular cells, which are expensive and difficult to culture.

Accordingly, in this study, we report a Transwell-based permeability assay that may have applications as a hemorrhagic activity test. First, the human microvascular endothelial cells (HMEC-1) culture conditions in culture inserts were examined, and the increase in the cell permeability in response to habu snake venom was then confirmed. Finally, in vitro assays were performed for detecting molecules that could neutralize the toxicity of habu snake venom. We also examined the effects of a serine protease inhibitor and metalloprotease inhibitor on neutralization of the toxicity of habu snake venom.

**Experimental**

**Habu snake venom**

Habu snake venom hemorrhagic factor-II (HR-2; National Institute of Infectious Diseases, Tokyo, Japan) was prepared at concentrations of 0.02 - 2.0 g L⁻¹, as estimated from a measurement of the absorbance at 280 nm with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The minimum hemorrhagic dose (MHD) of the venom was 29000 MHD mL⁻¹ (2.0 g L⁻¹ protein).

**Cells**

Immortalized human microvascular endothelial cells (HMEC-1 cells) were selected as a representative endothelial cell line. HMEC-1 cells were cultured in 25-cm² cell culture flasks (TrueLine, Nippon Genetics, Tokyo, Japan). The cells were grown in MCDB 131 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 30% fetal bovine serum (FBS; Invitrogen), 1µg Glutamax-I Supplement (Invitrogen), 13 µg mL⁻¹ hydrocortisone (Sigma-Aldrich, St. Louis, MO), and 10 ng mL⁻¹ epidermal growth factor (Sigma-Aldrich).

Once cells reached confluence, the medium in the cell culture flasks was aspirated. The cells were washed with 5 mL of phosphate-buffered saline (PBS; Takara Bio, Shiga, Japan) and then treated with 500 µL TrypLE Express (Invitrogen). After the cells were detached from the surface of the flask, 1 mL of fresh medium was added, and the obtained cell suspension was added to 4 mL of fresh medium in a 15-mL conical tube. The tube was centrifuged at 1200 rpm for 3 min, and the supernatant was aspirated. Finally, the cells were resuspended in medium at the appropriate concentration.

**Cell culture in wells**

Wells comprised of a coverslip and a 4-mm thick polydimethylsiloxane (PDMS) sheet (Silpot 184 W/C; Dow Corning Toray, Tokyo, Japan) having 5-mm inner diameter holes were used for live/dead cell assays to determine the effects of snake venom. The PDMS wells were coated with 10 µg mL⁻¹ human fibronectin (Corning, NY, USA) at 37°C for 1 h. Next, the cell culture medium was added to the well. After incubating with medium at 37°C for 30 min, 20 µL of the wells were introduced into the well. The wells were incubated at 37°C for 24 h in a 5% CO₂ incubator.

After culturing for 24 h, the cells were treated with 40 µL of a medium containing snake venom (0.02 g L⁻¹), and incubated at 37°C for 1 h. Cell viability assays were performed using two fluorescent dyes (LIVE/DEAD Viability/Cytotoxicity assay kit; Thermo Fisher Scientific). Cells were treated with 40 µL calcin-AM (2 µM) and ethidium homodimer (4 (µM) in PBS(+) for 30 min at 37°C under 5% CO₂ conditions, and then rinsed with PBS(+). Observations were carried out using a fluorescence microscope.

**Cell culture in Transwell cell culture inserts**

Twenty-four-well cell culture inserts with 1.0-µm pore PET membranes (Becton Dickinson, NJ, USA) were added to the bottom reservoir plates and coated with 200 µL of 10 µg mL⁻¹ human fibronectin (Corning) in PBS at 37°C for 1 h. Next, 100 µL of the fibronectin solution was removed, and 200 µL of culture medium was added to the insert (upper compartment). After incubating with the medium at 37°C for 30 min, 200 µL of the solution was removed, and 100 µL of the HMEC-1 suspension (1.0 × 10⁸ or 2.0 × 10⁸ cells mL⁻¹) was then added to the insert. Next, 500 µL of the culture medium was added into a reservoir of a 24-well plate (lower compartment). The plate with the inserts was incubated at 37°C for 24 h in a 5% CO₂ incubator. The next day, the cell culture inserts were transferred to another plate containing a fresh medium.

**Transepithelial electrical resistance (TEER) measurement**

A Millicell ERS-2 system (Merck Millipore, Billerica, MA, USA) was used for TEER measurements. First, the chopstick probe electrodes were sterilized with 70% ethanol for 15 min. After drying the ethanol, the electrodes were rinsed with the culture medium for a few seconds. The shorter electrode of the chopstick probe was placed into the upper compartment (culture insert) containing 500 µL culture medium, and the other electrode was placed in the lower compartment (24-well plate) containing 1000 µL medium. The probe was held steady and vertically relative to the plate insert.

**Permeation test**

The cells cultured in the cell culture insert for 24 h were used for permeation tests. Permeation tests were conducted by introducing 100 µL (culture insert) and 500 µL (24-well plate) of a culture medium containing 0.020, 0.20, or 2.0 g L⁻¹ habu snake venom. The concentration range corresponded with that used in rabbit assays. After incubation for 30 min, the medium in the cell culture insert was replaced with 300 µL of culture medium containing a fluorescent tracer, 40-kDa dextran-FITC (1.0 × 10⁻³ mol L⁻¹; Sigma-Aldrich). The cell culture inserts were then transferred to another bottom plate containing fresh medium. After incubation for 30 min in a CO₂ incubator, the fluorescence intensities of the medium in the cell culture inserts and 24-well plates were measured with a spectrofluorometer (FP-750; JASCO, Tokyo, Japan) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.
Inhibitor assay

The serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF; Sigma-Aldrich) and the metalloproteinase inhibitor Batimastat (BB-94; Merck Millipore) were used as protease inhibitors, and were added to the culture medium with 0.20 g L\(^{-1}\) habu snake venom. The concentrations of the inhibitors were 0.25 mM for AEBSF and 1.5 \(\mu\)M for BB-94, and samples were incubated for 2 h for AEBSF or 5 min for BB-94. After incubation, the culture medium containing the snake venom and inhibitor was used for permeation tests.

Ethylenediaminetetraacetic acid (EDTA) was also used as a metalloproteinase inhibitor. Two microliters of 10 \(\mu\)mol L\(^{-1}\) EDTA was added to 20 \(\mu\)L snake venom and incubated for 30 min. After incubation, EDTA was removed from the snake venom solution by ultrafiltration with an Amicon Ultra 0.5 mL 3000 MWCO centrifugal filter (Merck Millipore) before adding to the cells, because EDTA is a \(Ca^{2+}\) chelating agent and has cell detaching activity. The snake venom containing EDTA and 500 \(\mu\)L PBS were added to the Amicon device and centrifuged at 14,000g for 5 min. The resulting concentrated snake venom was diluted with 500 \(\mu\)L PBS and centrifuged again. The concentrated snake venom was collected in a new tube and diluted with culture medium to a volume of 2 mL. The culture medium containing the EDTA-treated snake venom was used for permeation tests.

Permeation coefficient

The permeability coefficient \(P\) was calculated from the following equation:\(^{21}\)

\[
P = \frac{\Delta C_L \times V_L}{C_U \times \Delta t \times A},
\]

(1)

where \(C_U\) is the initial fluorescence intensity of the medium in the cell culture insert (upper compartment), \(\Delta C_L\) is the fluorescence intensity change in the 24-well plate (lower compartment), \(V_L\) is the volume of the well (0.5 cm\(^3\)), \(A\) is the area of the membrane (0.3 cm\(^2\)) that allows fluorescent tracers to permeate from the culture insert to the well plate, and \(\Delta t\) is the assay time (1800 s). Lastly, the permeability of HMEC-1 cells, \(P_e\), was calculated from the following equation, where \(P_b\) is the permeability of the cell culture insert without cells:

\[
\frac{1}{P_e} = \frac{1}{P} - \frac{1}{P_b}.
\]

Microscopic observation

Cell images were obtained using an inverted fluorescent microscope IX71 (Olympus, Tokyo, Japan) equipped with objective lenses, UPlanFLN10 × Ph1 and LCPlanFLN20 × Ph1 (Olympus), a 100-W high-pressure mercury lamp, and a cooled CCD camera (ORCA-R2; Hamamatsu Photonics, Hamamatsu, Japan). For observation of ethidium homodimer, a dichroic mirror block (U-MWIG3; excitation 530 – 550 nm, emission >575 nm) was used. For the observation of calcein-AM, another dichroic mirror block (U-MNIBA3; excitation 470 – 495 nm, emission 510 – 550 nm) was used. Images were processed using HCImage (Hamamatsu Photonics) and ImageJ 1.45f software (National Institutes of Health, MD, USA).

Results and Discussion

Effects of the snake venom on cultured cells

Figure 1 shows the cell morphology before (Fig. 1a) and after a 30-min incubation with snake venom (protein concentration: 0.20 or 2.0 g L\(^{-1}\); Figs. 1b and 1c). As shown in Figs. 1e and 1f, most cells were alive (green) after the venom treatment. The snake venom did not induce immediate cell death. In contrast, cells began to round up, and cell detachment (Fig. 1b) and aggregation (Fig. 1c) were observed, in comparison with that before the venom treatment (Fig. 1a). Thus, the habu snake venom appeared to induce vascular damage by cell detachment prior to cell death.
Permeability test

The cell culture conditions in the cell culture inserts were examined. To examine the effects of the cell seeding density on the permeability of the HMEC-1 monolayer, permeability assays were performed with cell seeding densities of $1.0 \times 10^5$ or $2.0 \times 10^5$ cells cm$^{-2}$ in the 24-well cell culture inserts. The calculated permeability coefficient at $1.0 \times 10^5$ cells cm$^{-2}$ was $1.3 \pm 0.06 \times 10^{-5}$ cm s$^{-1}$ for 40-kDa FITC-dextran, and that at $2.0 \times 10^5$ cells cm$^{-2}$ was $3.4 \pm 0.14 \times 10^{-6}$ cm s$^{-1}$. These values were comparable with those reported for the HUVEC monolayer$^{22}$ for 4-kDa dextran ($5.5 \pm 1.3 \times 10^{-6}$ cm s$^{-1}$) and for 20-kDa dextran ($3.8 \pm 0.4 \times 10^{-6}$ cm s$^{-1}$). Because the lower permeability was preferable, a cell seeding density of $2.0 \times 10^5$ cells cm$^{-2}$ was selected for subsequent experiments.

Next, permeability assays were performed using culture medium containing 0.020, 0.20, or 2.0 g L$^{-1}$ habu snake venom. The permeability coefficients of HMEC-1 were increased by increasing the concentration of habu snake venom (Fig. 2). As shown in Figs. 1b and 1c, the cell monolayer was damaged by stimulation with the habu snake venom, and enlargement of the holes in the monolayer led to rupture. Taken together, the permeability coefficients of the HMEC-1 monolayer were increased by increasing hole areas in the monolayer.

Effects of inhibitors

Snake venom induced-hemorrhage is thought to occur primarily through multiple metalloproteinases.$^{25}$ HR2, a 23-kDa metalloproteinase, is a major component of habu snake venom.$^{26}$ Therefore, we evaluated the effects of protease inhibitors on habu snake venom stimulation.

First, we assayed the serine protease inhibitor AEBSF. Notably, however, habu snake venom activity was not reduced by the AEBSF treatment, and the HMEC-1 monolayer was damaged. The permeability coefficient of the HMEC-1 monolayer with the AEBSF-treated habu snake venom was almost the same as that of untreated habu snake venom (Fig. 3a). Thus, these results showed that AEBSF did not work as an inhibitor of habu snake venom. These results indicate that HR2 is a metalloproteinase.

Next, we examined the matrix metalloproteinase inhibitor Batimastat (BB-94). In contrast to AEBSF, the BB-94 treatment reduced habu snake venom activity and prevented damage to the HMEC-1 monolayer. The permeability coefficient of the HMEC-1 monolayer with BB-94-treated habu snake venom was $72 \pm 5.1 \times 10^{-6}$ cm s$^{-1}$, which is lower than that of the untreated habu snake venom (240 $\pm 25.0 \times 10^{-6}$ cm s$^{-1}$; Fig. 3b). Additionally, when the concentration of BB-94 was changed from 1.5 to 15 μM, the higher concentration resulted in a lower permeability coefficient ($52 \pm 5.4 \times 10^{-6}$ cm s$^{-1}$ for 15 μM; $p = 0.07$). Thus, these data showed that BB-94 acted as an inhibitor of habu snake venom.

Finally, the metal chelator and metal-dependent protease inhibitor EDTA was assayed. Because EDTA induces cell detachment, EDTA was removed from the venom by ultrafiltration before adding the EDTA-treated habu snake venom to the cell culture medium. Notably, the EDTA treatment
reduced habu snake venom activity. The permeability coefficient of the HMEC-1 monolayer with EDTA-treated habu snake venom was 14 ± 2.8 × 10⁻⁶ cm s⁻¹, which was lower than that of untreated habu snake venom (101 ± 10.7 × 10⁻⁶ cm s⁻¹; Fig. 3c). Consistent with these findings, BB-94 and EDTA are known to inhibit the 24-kDa hemorrhagic metalloproteinase isolated from Bothrops asper.²⁰,²⁷

Conclusions

In this study, we developed a new snake venom toxicity assay based on rapid and multiplexed Transwell-based analysis of changes in the permeability of the endothelial cell monolayer induced by snake venom. This fluorescent permeability assay was advantageous in that it reduced the assay time from 24 h for animal tests to 2 h, and that many samples could be assayed easily. Moreover, in the Transwell assay, the increase in permeability caused rupturing of the HMEC-1 monolayer induced by snake venom. This assay was based on the proteolytic degradation of capillary basement membrane proteins and venom induced-vascular permeability, which both occur in response to snakebites in vivo. Thus, this assay may be an alternative to conventional animal hemorrhage tests, and could be used for the in vitro analysis of blood vessel injury and snake venom neutralizers. Finally, the assay established in this study may facilitate the development of new therapeutic agents for the treatment of human diseases and disorders, particularly those related to effects on human microvascular endothelial cells, which were used in the assay.

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