Detection of Mast Cells Expressing c-Kit Using Antibody Covalently Bound to Gelatin Elongated from Surface of Immunosensor Based on Surface Plasmon Resonance

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An immunosensor based on surface plasmon resonance was applied to detect mast cells expressing c-Kit. Sufficient detection of the mast cells was achieved by covalent immobilization of gelatin firstly on the sensor surface and followed by covalent binding of the anti-c-Kit antibody to lysine residues in the gelatin molecules through bis(sulfosuccinimidyl)-suberate (BS3) treatment. By using BS3, which is a homo-bifunctional reagent, the lysine residues of the anti-c-Kit antibody easily bound to the lysine residues of the gelatin in the physiological condition. The lower limit of detection was 10⁴ cells/mL.

Keywords SPR, immunosensor, bone marrow-derived mast cells, c-Kit, antibody, gelatin

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Introduction

A c-Kit is one of the tyrosine kinase receptors that are expressed on the surface of many types of cells, including hematopoietic progenitor stem cells, mast cells, melanocytes, interstitial cells of Cajal, and germ cells.1,6 A stem cell factor binds to the c-Kit and plays an important role in cell development.1 Although the normal cells often lose the c-Kit with maturation, mast cells continue to express the c-Kit after maturation.3,7,8 Since the c-Kit is constantly and strongly expressed on the surface of mast cells, it is a good cell marker.9–11

The mast cells play a key role in IgE-associated immune responses.5,8 After the allergen is bound to the IgE, of which Fc domain had been bound to FcεRI receptor of the mast cell surface, chemical mediators such as histamine, leukotriene, and prostaglandin are released from the granules. They cause inflammatory responses as the result. It is therefore important to detect mast cells for research works of such a type I hypersensitivity.

The mast cells are generally detected by using a microscope or a flow cytomter after reacting with fluorescence-labeled anti-c-Kit antibody.9,11 On the other hand, an immunosensor based on surface plasmon resonance (SPR-immunosensor) was developed for real-time detection of the MEG01s cell line expressing c-Kit derived from human fetal kidney, without fluorescence label.12 In this study, the mast cell however almost cannot be detected by their condition. The purpose of this study is to determine the adequate condition of the SPR-immunosensor to detect the mast cells.

It was described that the mast cells were successfully detected by covalent immobilization of gelatin firstly on the sensor surface and followed by covalent binding of the anti-c-Kit antibody to lysine residues in the gelatin through bis(sulfosuccinimidyl)suberate (BS3) treatment. By using BS3, which is a homo-bifunctional reagent, the lysine residues of the anti-c-Kit antibody were easily bound to the lysine residues of the gelatin in the physiological condition.

Experimental

Materials

Anti-c-Kit polyclonal antibody (goat IgG) reacted with human and mouse c-Kit was purchased from R&D Systems, Inc. (Minnesota, USA). Nonspecific antibody (goat IgG) was purchased from Abcam plc (Cambridge, UK). RPMI-1640 medium, and nonessential amino acids were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Hyclone Laboratories (South Logan, UT, USA). L-Glutamine solution (200 mmol/L) was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Penicillin and streptomycin sulfate were purchased from Life Technologies Corporation (Grand Island, NY, USA). BS3 was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Gelatin (fine powder), Dulbecco’s
phosphate-buffered saline without calcium and magnesium (PBS), and all other chemicals of analytical grade were purchased from Nacalai Tesque (Kyoto, Japan).

**Preparation of mast cells**

The mouse bone marrow-derived mast cells were prepared based on the method described by Kanaoka et al. Briefly, bone marrow cells were collected from femurs and tibiae of the female reticulated Balb/c mouse. They were cultured for 4-8 weeks in RPMI-1640 medium containing 10% of FBS, L-glutamine (2 mmol/L), nonessential amino acids (0.1 mmol/L), penicillin (100 units/mL), streptomycin (100 μg/mL), and 1% of cultured supernatant from Chinese hamster ovary cells secreting mouse interleukin-3. The culture medium was changed every week, and the cell density was adjusted to 3×10⁶ cells/mL by each passage. After 4 weeks, it was confirmed that more than 97% of the cells became the mast cells, by staining with Eosinophil-Mast Cell Stain Kit from ScyTek Laboratories, Inc. (Utah, USA).

The mouse experiment was performed under the guidelines of the Animal Experiment Committee of Horiba Ltd.

**Immunosensor construction**

The surface plasmon resonance imaging system (OpenPlex, Horiba Scientific, Palaiseau, France), and the sensor chip consisting of a glass prism and gold thin layer modified with the carboxy group esterified by N-hydroxysuccinimide (NHS) (SPRi-Biochip CS-HD, Horiba Scientific) were used for immunoasnsensor construction.

The gelatin solution (1.0 mg/mL) was coated onto the sensor chip surface and covalently bound to the carboxy group through its NHS upon immediate standing at 25°C for 16 h in 80% relative humidity. After washing 3 times with distilled water, BS3 solutions that had been diluted to 0.1, 1, and 10 mmol/L in PBS were spotted onto the chip surface (12×23 mm) at each 10 nL/spot (500 μm diameter) by using a spotter (Spot Master, Musashi Engineering, Tokyo, Japan), which had been tuned for the SPRi-Biochip. This spotter can spot easily at the same position by positioning the center, X axis, and Y axis of the sensor chip in the dedicated chamber. It was immediately left to stand at 25°C for 1 h in 80% relative humidity. After washing 3 times with distilled water, anti-c-Kit antibody and nonspecific antibody solutions (0.2 mg/mL) were respectively spotted on the same position as the above BS3 solutions and washed immediately left to stand at 25°C for 16 h in 80% relative humidity. After washing with distilled water, it was blocked with PBS with the gelatin solution (10 mg/mL) at 25°C for 1 h. The residual NHS group on the chip and in the BS3 were deactivated with ethanolamine solution (1 mol/L, adjusted to pH 8.5) for 30 min, and then washed with PBS.

Meanwhile, the antibodies were covalently immobilized to the carboxy group of the sensor chip surface through NHS, as described previously. In brief, the anti-c-Kit antibody and the nonspecific antibody were diluted to 0.2 mg/mL in PBS with the gelatin solution (1.0 mg/mL). The antibody solutions were spotted onto the chip surface using the above spotter and was immediately left to stand at 4°C for 16 h in 80% relative humidity. After washing with distilled water, the antibody bound sensor chip was treated by following the same procedure as described above.

Antibody immobilized sensor chips were respectively set into the instrument to construct the immunoasnor. The sensor chip was rinsed with the running buffer, PBS with gelatin (1.0 mg/mL) and Tween20 (200 μg/mL) at 25 µL/min until the SPR signal was stabilized.

**Detection of mast cells expressing c-Kit**

The mast cell suspensions were prepared at 1×10⁶, 1×10⁷, and 1×10⁸ cells/mL with the running buffer and each 200 µL was injected to the immunoasnsensor, and flowed at 25 µL/min for 480 s. It was continuously changed to the running buffer and was further flowed at 25 µL/min for 700 s. After detection of the signal from the bound cells, the sensor chip was regenerated with roughly crushed gelatin gel (30 mg/mL) at 25 µL/min for 480 s, as described previously. The sensor chip was reused for the next detection.

**Results and Discussion**

Detection of mast cells expressing c-Kit was firstly examined by using the anti-c-Kit antibody directly immobilized to the sensor surface in the condition without BS3 treatment, as illustrated in Fig. 1A. However, the SPR signal from the mast cells was not found in this condition except for the slight signal found as two white dots by injection of 1×10⁷ cells/mL, as shown in Fig. 2A. Because all of the mast cells should constantly express much c-Kit molecules, the result was contrary to our expectation.

One of the reasons might be the difference of their cell sizes. The mast cells used were actually much bigger than MEG01s and HEK293T cells, which had been detected in this condition as described previously. The SPR image was therefore observed as dots by CCD camera, as shown in Fig. 2A, and differed from MEG01s and HEK293T smear images, which mean that their cell sizes were smaller than the resolution limit. Another one might be the difference of the reaction conditions between the SPR-immunosensor and the conventional fluorescence-labeled anti-c-Kit antibody technique. The latter would allow for free reaction with the anti-c-Kit antibody to the c-Kit of the cell surface in the liquid phase, while the former might hardly allow for the anchoring of the big mast cells against the liquid flow on the flat and solid phase due to reaction with the immobilized antibody.

As a working hypothesis to increase the bound mast cells, it was proposed to immobilize the antibody at the position away from the sensor surface, at the range where an evanescent wave can reach. Gelatin, which is a soluble and fibrous protein...
denatured from collagen, contains lysine (4.1 to 4.7 g) in its dry weight (100 g) and was thus adopted for this material.\(^{15}\) After the gelatin was immobilized on the sensor chip, BS3, which is the protein cross-linker, was spotted to the gelatin at a concentration of 1.0 mmol/L from which the cross-linking between gelatin molecules was barely derived as described below. The anti-c-Kit antibody was covalently bound to the gelatin as illustrated in Fig. 1B. When the prepared sensor chip was used for the detection of the mast cells, 10^4 cell/mL of the mast cells could be sufficiently detected, as shown in Fig. 2B. The sensitivity of the condition with BS3 treatment improved more than 100 fold compared to the condition without BS3 treatment.

The concentration of BS3 would be the most critical to this performance among the three materials for the sensor chip preparation. It was roughly estimated that the immobilized gelatin would be 20 fmol calculated from the adsorption amounts on the saturated condition by each spot and then the lysine residue numbers would be 0.6 pmol/spot. When the BS3 was prepared at different concentrations, 1.0 mmol/L (17 pmol/spot) was the optimum as shown in Fig. 3. The ratio of the lysine residue and the BS3 was roughly estimated as 30 fold. The BS3 (0.1 mmol/L: 1.7 pmol/spot) took the same strength of the signal but the bound mast cells were more quickly released from the antibody after the cell supply finished. On the other hand, the BS3 (10 mmol/L: 170 pmol/spot) took less strength of the signal and D. Irikura is a current employee of Horiba Ltd., which is the maker of the SPR imaging system used in this study.

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