Evaluation of the Anti-Allergic Effect of Natural Medicines on Mast Cell by Using Two-Dimensional Surface Plasmon Resonance Observation

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ABSTRACT
Demand for novel drug screening methods which are characterized by simple, rapid and low cost is increasing now. In this study, the simple and rapid evaluation of the anti-allergic effect of natural medicines on the degranulation of a model mast cell (RBL-2H3 cells) was successfully achieved by two-dimensional surface plasmon resonance (2D-SPR) measurement. The each cell response was observed by SPR imaging upon model antigen, Albumin from bovine serum, 2, 4-Dintrophenylated (DNP-BSA) stimulation after anti-DNP IgE sensitization on RBL-2H3 cells. Here glycyrrhizic acid (GA) and isoliquiritigenin (ISL) were examined as degranulation inhibitors. After the pretreatment with 30 μmol/L GA or 50 μmol/L ISL, the reflection intensity increase at cell regions upon DNP-BSA stimulation was completely suppressed. In addition, it was shown that the suppression of reflection intensity increase at cell regions in the 2D-SPR observation upon DNP-BSA stimulation was dependent on the GA and ISL concentration in pretreatment as well as β-hexosaminidase assay. These results demonstrated that the suppression effect of GA and ISL on the degranulation of RBL-2H3 cells could be evaluated by 2D-SPR observation at cell regions in quick and simple manner. Our study further suggested that 2D-SPR observation of mast cell response might be applicable to screen anti-allergic components of natural products and useful to discuss its inhibitory effect in the intracellular signaling pathway of mast cell upon antigen stimulation.

1. Introduction
Search for natural medicines possessing anti-allergic activity has contributed to treatment of allergic diseases such as rhinitis, pollinosis, dermatitis, bronchial asthma and so on.¹ There are various kinds of methods for screening compounds which have anti-allergic activity such as enzyme-based, receptor-based and cell-based assay.²–⁴ However, these conventional methods have common disadvantages of high cost, time consuming and complex treatment in the screening process. Thus the more rapid, simple and low cost methods have been required in screening nature medicines. The rat basophilic leukemia cell (RBL-2H3) is wildly used as a model mast cell in allergy studies. The antigen stimulation causes degranulation in RBL-2H3 cells and release of histamine and β-hexosaminidase. Usually, the degranulation response in mast cells is confirmed by HPLC analysis of the released histamine or enzymatic activity of the released β-hexosaminidase.

On this background, Hide and Yanase et al. had applied a surface plasmon resonance (SPR) sensor for the first time to monitor the physiological response in RBL-2H3 cells upon antigen stimulation.⁵–⁷ After their frontier studies, we have tried to monitor and quantify the allergic response of RBL-2H3 cells by two-dimensional surface plasmon resonance (2D-SPR) measurement at single cell level.⁸⁻¹⁰ Our results demonstrated that 2D-SPR observation was a highly-sensitive, real-time and reagent-less method for monitoring of the allergic response at individual RBL-2H3 cells and available for antigen screening and quantification. We have also speculated that the 2D-SPR response by the local refractive index change at RBL-2H3 cell bottom upon antigen stimulation might be related with protein kinase C (PKC) translocation in each RBL-2H3 cell as well-considered by Hide et al.¹⁰ It has been already known that the antigen activates the FcεRIIs by the cross-linking of IgE molecules bound on the FcεRIIs and the intracellular signal pathway inducing the PKC translocation and degranulation in mast cells.¹¹ In this study, next we applied 2D-SPR observation to evaluate the anti-allergic effect of natural medicines on mast cell. We chose glycyrrhizic acid (GA) and isoliquiritigenin (ISL) that are the natural components from Liquorice as model natural medicines. Because they are the most famous degranulation inhibitors on mast cells. And so many researchers have studied and published many reference papers about the anti-allergic activity of GA and ISL.¹²,¹³ GA and ISL can inhibit the IgE-mediated degranulation in mast cell through the blocking of the Ca²⁺ influx.¹⁴ The other reference paper further suggested that the inhibition of Ca²⁺ concentration increase contributed to the suppression of the PKC translocation.¹⁵ Based on these data, we expected that GA and ISL could suppress PKC (α and β) translocation and following degranulation in RBL-2H3 cells upon antigen stimulation and the suppression of the intracellular reaction might be simply monitored by 2D-SPR observation. Our study may provide a very useful method for screening anti-allergic components of natural products.

2. Experimental
2.1 Materials
RBL-2H3 cell was obtained from the cell bank of RIKEN BioResource Center (Tsukuba, Japan). Eagle minimal essential medium (EMEM) and penicillin/streptomycin were purchased from Gibco (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Biowest (Nuaille, France). Albumin from bovine serum, 2,4-
Dintrophenylated (DNP-BSA) was purchased from Merck Millipore (Massachusetts, USA). Hanks’ balanced salts (HBSS), anti-DNP IgE and p-nitrophenyl-N-acetyl-β-D-glucosaminide were obtained from Sigma-Aldrich Japan (Tokyo, Japan). Hanks’ balanced salts solution (HBSS) was prepared by dissolving HBS powder to ultrapure water. The 50 nm gold thin film-coated high refractive index glass (SF6) chip (18 × 17 mm) was purchased from ALS Co., Ltd. (Tokyo, Japan), and flexiPERM (11 × 7 × 10 mm) was obtained from Greiner Bio-One (Germany), Glycyrrhizic acid (GA), isoliquiritigenin (ISL) and sphingosine were purchased from Wako (Osaka, Japan).

2.2 Cell culture
RBL-2H3 cells were maintained in EMEM supplemented with FBS (10%), penicillin/streptomycin (1%) in a cell culture flask (25 cm²) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Before experiments, the RBL-2H3 cells (1 × 10⁵ cells in 300 µL) were reseeded on a 50 nm gold thin film-coated high refractive index glass (SF6) chip with a rectangular well of flexiPERM (11 × 7 × 10 mm) for one day (24 h). Prior to 2D-SPR observation, the culture medium in the RBL-2H3 cell adhered chip chamber was replaced with HBSS (pH 7.4, 37°C) and experiments were then conducted under room temperature.

2.3 2D-SPR observation of cell regions and analysis
The 2D-SPR instrument (2D-SPR04A, NTT-AT, Japan) with a collimator lens for parallelizing incident light and a cooling CCD camera coupled with four kinds of magnification lens (1×, 2×, 4× and 7×) was used to observe the reflection image and to monitor the time-course of reflection intensity at the region of interests. Prior to 2D-SPR observation, the culture medium in the cell chip chamber was removed and washed with 300 µL HBSS (pH 7.4, 37°C) and then the RBL-2H3 cells were sensitized with 300 µL of HBSS including 0.1 µg/mL anti-DNP IgE for 1 h. For testing the GA and ISL, 300 µL of HBSS including 30 µmol/L GA or 50 µmol/L ISL was poured into the cell chip chamber and incubated for 30 min after the sensitization with anti-DNP IgE and washing process. After pre-incubation with or without GA or ISL, the cell chip solution was replaced with pure HBSS, and the cell chip was placed on the top of the prism of the 2D-SPR instrument with refractive index matching oil to avoid undesirable reflection light. The 2D-SPR measurement was then conducted under room temperature. The SPR angle of each RBL-2H3 cell region was measured by monitoring of SPR angle at initial resonance angle of 51.5° to 52.0° and the maximum change in reflection intensity at each cell region and a gold region at 3.2 Evaluation of anti-allergic effect of GA on RBL-2H3 cell by 2D-SPR measurement
To evaluate the anti-allergic effect of GA on model mast cells by 2D-SPR observation, the resonance angle and the optimal measurement angle for time-course monitoring of reflection intensity was investigated at first. The SPR angle was measured by monitoring of the reflection intensity at a same cell region and a gold region at various incident angles (from 49° to 55° every 0.1°). As shown in Fig. S1, after DNP-BSA stimulation, the resonance angle shifted from 51.5° to 52.0° and the maximum change in reflection intensity before and after DNP-BSA stimulation was seen at 50.9°. Therefore, the optimum incident angle for monitoring the RBL-2H3 cell response was determined at 50.9°.

2.4 β-Hexosaminidase assay
Degranulation of same number of RBL-2H3 cells was evaluated from the enzyme activity of released β-hexosaminidase from RBL-2H3 cells. The degranulation inhibition effects of GA and ISL were also evaluated by the enzyme activity of β-hexosaminidase released from RBL-2H3 cells upon same concentration of antigen stimulation after pretreatment of GA or ISL.11 Briefly, RBL-2H3 cells were dispensed into 36 wells in a 96-well plate at a concentration of 5 × 10⁴ cells/well using EMEM containing 0.45 µg/mL of anti-DNP IgE and incubated for 1 h at 37°C in 5% CO₂ for IgE sensitization of the cells. The RBL-2H3 cells were washed twice with HBSS (pH 7.4, 37°C), and then the solution was replaced with 80 µL of HBSS containing 0.1% BSA for blocking. 10 µL of HBSS containing GA or ISL was added into each well and the cells were incubated for 30 min. Then the well wash was again with HBSS and followed by the addition of 10 µL of HBSS containing various concentration of DNP-BSA at 37°C for 10 min to induce the degranulation of RBL-2H3 cells. The experiment in the same condition was done for three times. The degranulation reaction was stopped quickly by cooling the well plate in an ice bath for 10 min. 30 µL supernatant in each well was then transferred to the empty wells in a new 96-well plate and 70 µL of p-nitrophenyl-N-acetyl-β-D-glucosaminide solution (1 mg/mL in 0.1 mol/L citrate buffer solution, pH 4.5) was added to each well solution to start enzymatic reaction and kept at 37°C for 1 h. The enzymatic reaction was stopped by adding 200 µL of stop solution (0.1 mol/L Na₂CO₃/ NaHCO₃, pH 10.0). The absorbance of each well solution was then measured with a microplate reader at 405 nm (BMGLABTECH, Japan) to calculate the enzyme activity of released β-hexosaminidase. The relative ratio of the released β-hexosaminidase activity was used to evaluate the degranulation inhibition effect by GA and ISL.

2.5 Investigation of SPR response mechanism
The cell culture and 2D-SPR experimental procedure are similar as described above. 300 µL of HBSS including 10 µmol/L of sphingosine was poured into the cell chip chamber and incubated for 30 min after the sensitization with anti-DNP IgE and washing process. For the 2D-SPR observation of cell response, HBSS (10 µL) was first injected at 3 min for negative control stimulation and then 10 µL of DNP-BSA solution (10 ng/mL in HBSS) was injected at 6 min to induce the degranulation of the RBL-2H3 cells.

3. Results and Discussion
3.1 Resonance angle measurement and optimal angle determination for 2D-SPR observation in RBL-2H3 cells
To evaluate the anti-allergic effect of GA on model mast cells by 2D-SPR observation, the resonance angle and the optimal measurement angle for time-course monitoring of reflection intensity was investigated at first. The SPR angle was measured by monitoring of the reflection intensity at a same cell region and a gold region at various incident angles (from 49° to 55° every 0.1°). As shown in Fig. S1, after DNP-BSA stimulation, the resonance angle shifted from 51.5° to 52.0° and the maximum change in reflection intensity before and after DNP-BSA stimulation was seen at 50.9°. Therefore, the optimum incident angle for monitoring the RBL-2H3 cell response was determined at 50.9°.

3.2 Evaluation of anti-allergic effect of GA on RBL 2H3 cell by 2D-SPR measurement
The RBL-2H3 cell response upon DNP-BSA (10 ng/mL) stimulation was next observed with the 2D-SPR instrument. Before the 2D-SPR observation, the culture medium in cell chip chamber was replaced with HBSS. At the measurement angle, the reflection intensity at each cell region was monitored and recorded by the 2D-SPR instrument. HBSS as a negative control and DNP-BSA were injected consecutively at 3 min and 6 min, respectively. The reflection intensity was significantly increased after DNP-BSA stimulation at each cell region as shown in Fig. 1A, but no significant change was observed at a gold region. The time-course measurement demonstrated that the reflection intensity increased slowly by the antigen stimulation. The SPR images of RBL-2H3 cell regions changed brightly 10 min after the stimulation as shown in Figs. S2A and S2B.
To evaluate anti-allergic effect of natural compounds by using 2D-SPR observation in RBL-2H3 cells, GA was used initially as a degranulation inhibitor. The IgE sensitized RBL-2H3 cells were incubated in HBSS containing 30 µmol/L GA for 30 min. After incubation and washing, the RBL-2H3 cell response upon antigen stimulation was monitored with the 2D-SPR instrument and yielding reflection intensity data was shown in Fig. 1B. The HBSS was injected at 3 min and DNP-BSA was administrated at 6 min. The reflection intensity was not changed after the DNP-BSA stimulation at four cell regions. In the SPR images, the brightness of cell regions changed little before and after stimulation as shown in Figs. S2C and S2D. Our previous work8,9 and the supporting data (Fig. S2) showed that the reflection intensity increase was obviously involved with the degranulation response of RBL-2H3 cell upon DNP-BSA stimulation. No change of the reflection intensity upon antigen stimulation after GA pretreatment suggested that GA has enough inhibition effect on the intracellular signaling in RBL-2H3 cells for degranulation by antigen stimulation.

In addition, the inhibition effect of GA on RBL-2H3 cell allergic response was examined in the concentration range of lower than 30 µmol/L. The change of time-course of reflection intensity upon antigen stimulation for various concentration of GA was recorded and shown in Fig. 2A. GA-concentration dependence of the average of reflection intensity increase at five cell regions was shown in Fig. 2B. The IC$_{50}$ of GA was estimated to be 2 µmol/L.

On the other hand, GA-concentration dependence of the relative enzyme activity of β-hexosaminidase released from cells by the DNP-BSA stimulation was shown in Fig. 2C. The experiment in the same condition was done three times. The IC$_{50}$ of GA was estimated to be 6 µmol/L by the β-hexosaminidase assay. From the comparison of 2D-SPR evaluation and β-hexosaminidase assay, it was suggested that 2D-SPR observation was applicable for evaluating anti-allergic effect of GA as well as β-hexosaminidase assay.

3.3 Evaluation of the anti-allergic effect of ISL on RBL 2H3 cell by 2D-SPR measurement

Next we used isoliquiritigenin (ISL) as another degranulation inhibitor. The experimental method was same as described in 3.2. Without ISL pretreatment, HBSS injection at 3 min showed no change of the reflection intensity, but DNP-BSA injection at 6 min induced significant increase of the reflection intensity as shown in Fig. 3A. However, after 30 min pretreatment of 30 µmol/L ISL, the reflection intensity at four cell regions showed almost no change.
upon the DNP-BSA stimulation as shown in Fig. 3B. These data suggested that 2D-SPR measurement succeeded to monitor the inhibition effect of ISL on the degranulation reactions in RBL-2H3 cells.

Furthermore, the ISL-concentration dependent suppression of reflection intensity increase in 2D-SPR measurement was shown in Fig. 4A. ISL worked as a dose-dependent inhibitor in the range lower than 50 µmol/L as shown in Fig. 4B. IC$_{50}$ of ISL was estimated to be 8 µmol/L. On the other hand, ISL-concentration dependence of the relative activity of β-hexosaminidase released from cells upon DNP-BSA stimulation was shown in Fig. 4C. The experiment in the same condition was done three times. The IC$_{50}$ of ISL was estimated to be 14 µmol/L by the β-hexosaminidase assay. The IC$_{50}$ of ISL by our β-hexosaminidase assay was slightly smaller than that was reported by another researchers. From these data, it was demonstrated that the 2D-SPR observation could successfully evaluate the degranulation inhibition effect of ISL on mast cells.

3.4 Discussion of SPR response mechanism

The 2D-SPR observation is very sensitive and able to monitor a very small change of refractive index in the evanescent field (less than the wavelength of incident light) at cell bottom. We have hypothesized that PKC (α and β) translocation in RBL-2H3 cells might be involved in the reflection intensity change as previously reported by Hide et al. Therefore, 10 µmol/L sphingosine as a PKC specific inhibitor was administrated to RBL-2H3 cells for 30 min in pretreatment process before DNP-BSA stimulation and the reflection intensity change was monitored by the 2D-SPR instrument to discuss the SPR response mechanism. The SPR response was clearly suppressed by sphingosine pretreatment as shown in Fig. 5. This result indicated that PKC translocation certainly contributed to the reflection intensity change. Even though the detail mechanism of inhibition effect by GA and ISL against PKC translocation was still

Figure 3. A: Time-course of the reflection intensity increase at 4 cell regions in 2D-SPR observation upon 10 ng/mL DNP-BSA stimulation. B: Time-course of the reflection intensity at 4 cell regions in 2D-SPR observation upon 10 ng/mL DNP-BSA stimulation with pretreatment of 50 µmol/L ISL. 2D-SPR measurement angle was set at 50.9°.

Figure 4. A: The change of time-course of reflection intensity at a cell region upon 10 ng/mL DNP-BSA stimulation after pretreatment with various concentration of ISL. The ISL concentration of pretreatment were 0 µmol/L (---), 5 µmol/L (--·--·--·), 10 µmol/L (····), 20 µmol/L (···), 30 µmol/L (----), 50 µmol/L (——). B: ISL concentration dependence of the average of reflection intensity increase at 5 cell regions upon DNP-BSA stimulation. The reflection intensity change was measured 10 min after the antigen stimulation. C: ISL concentration dependence of the relative activity of β-hexosaminidase released from cells upon 10 ng/mL DNP-BSA stimulation. The results are expressed as the mean ± SD from three experiments.

Figure 5. Time-course of the reflection intensity at 4 cell regions upon 10 ng/mL DNP-BSA stimulation in 2D-SPR observation with the pretreatment of 10 µmol/L sphingosine. The reflection intensity at individual cell regions did not increase after DNP-BSA stimulation by the sphingosine pretreatment.
unclear, our results and reference data\textsuperscript{14–17} suggested that GA and ISL might inhibit the upstream of PKC translocation inducing the degranulation.

4. Conclusion

We have successfully achieved to evaluate the anti-allergic effect of traditional natural medicines by using a high resolution 2D-SPR instrument in quick and simple manner. The anti-allergic effect of GA and ISL on RBL-2H3 cells was evaluated by the suppression of reflection intensity increase at individual cell regions upon the antigen (DNP-BSA) stimulation. The experimental steps of 2D-SPR observation are less than B-hexosaminidase assay and did not use any reagents for degranulation analysis. The 2D-SPR observation of cell response need less than 1 hour. On the other hand, B-hexosaminidase assay needs more than 3 hours. Therefore, the mast cell-based 2D-SPR measurement is not only sensitive like B-hexosaminidase assay for screening natural medicines, but also easy, low cost and time saving. Our study suggested that 2D-SPR method might be promising to screen anti-allergic components of natural products and also useful to discuss the intracellular signaling pathway and inhibition mechanism in individual cells.

Supporting Information

The Supporting Information is available on the website at DOI: https://doi.org/10.5796/electrochemistry.20-00113.

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