Evaluation of Effects of Natural Medicines on Mammalian Cells by Using Two-Dimensional Surface Plasmon Resonance Observation

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Finally, I would like to thank all members of my laboratory, for they are kind enough to help and support me with everyday life in Japan.

Abstract

Natural products have various biological activities such as anti-allergic effect and anti-cancer effect, these excellent properties make them attractive as lead chemicals for discovery of new drugs. Screening of natural products is becoming more important to find new and effective drug compounds. Therefore, lots of methods such as colorimetric, fluorescence, bioluminescence, biological and cell-based assays were applied in screening drugs from natural products. However the high cost, time consuming and complex experimental steps were disadvantages of these methods. Thus, the necessary of more rapid, simple screening methods is increasing now. Recently, surface plasmon resonance (SPR) sensor has been applied in drug screening. SPR change has been used to monitor changes of the refractive index by specific binding reactions such as antigen-antibody binding and DNA hybridization on the surface of a gold chip. On the other hand, Prof. Shinohara's group has tried to monitor cell response to agonist stimulation by two-dimensional surface plasmon resonance (2D-SPR) measurement at single cell level. His group has already succeeded to monitor the cell response upon antigen stimulation on the IgE-sensitized model mast cell, RBL-2H3 cell as described in Chapter 1. It was further considered 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. These research background and purpose of this study was described in Chapter 1.

In Chapter 2, all materials and methods for this study were described.

In Chapter 3, two-dimensional surface plasmon resonance (2D-SPR) measurement as a simple and rapid method was successfully applied to evaluate the anti-allergic effect of natural medicines on the degranulation of RBL-2H3 cells. The each cell response was observed by SPR imaging upon stimulation with model antigen, Albumin from bovine serum, 2, 4-Dintrophenylated (DNP-BSA) after anti-DNP IgE sensitization on RBL-2H3 cells. Glycyrrhizic acid (GA) and isoliquiritigenin (ISL) from Licorice were examined as degranulation inhibitors. After the pretreatment with 30 μM GA or 50 μM ISL, the reflection intensity increase at cell regions upon DNP-BSA stimulation was completely suppressed. The suppression of reflection intensity increase at each cell regions in the 2D-SPR observation upon DNP-BSA stimulation was dependent on the GA and ISL concentration in pretreatment as same 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. My study further suggested that 2D-SPR observation 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.

In Chapter 4, I have tried to apply 2D-SPR observation to evaluate the anti-proliferative activity of ISL on human lung cancer cell (A549 cells). It is known that ISL exhibits an inhibitory effect on proliferative activity of cells in prostate cancer, hepatocellular carcinoma, breast cancer, melanoma, lung cancer and so on. The human lung cancer cell proliferation was suppressed with the ISL treatment over

100 μM. It was demonstrated that the SPR angle at A549 cell regions shifted positively day by day with cell proliferation in the absence of ISL, but the SPR angle did not change even after a few days in the presence of 100 μM ISL. It suggested that 2D-SPR observation could monitor human lung cancer cell proliferation and evaluate the anti-proliferation effect of ISL without any probes.

In Chapter 5, I concluded the usefulness and advantages of 2D-SPR observation for evaluation of anti-allergic and anti-proliferation effect of traditional natural medicines on mammalian cells. My study further suggested that 2D-SPR observation method might be useful to discuss the intracellular signaling pathway and inhibition mechanism in individual cells.

Contents

Chapter 1

Introduction

1-1 Natural medicine and drug screening

Natural medicines have played important roles in therapy of diseases and maintaining good health for human life. Some of natural medicines still have been used very well for therapy of diseases in the world. There are about 200,000 natural products have been identified from medicinal plants, animals and microorganisms.[1, 2] Therefore, natural medicines and their bioactive molecules are always in requirement and a central point of research. Natural products have various different chemical structures and bioactivities. Therefore, these chemical diversities as lead structures make them attractive for discovering new drugs.[3]

Screening of natural product is becoming more important to find new and effective drug compounds. Therefore, common assays such as colorimetric, fluorescence, bioluminescence; biological assays and cell-base assay were applied in screening natural products.[4-8] However, some disadvantages like high cost, time consuming and complex experimental steps effected the application of these methods. Thus, findings of a more rapid, simple and time-saving methods for drug screening became more important in recent years.

1-2 What is 2D-SPR

Surface plasmon resonance (SPR) is a phenomenon in which the electrons in the

metal surface layer are excited by photons of incident light with a certain angle of incidence, and then propagate in parallel to the metal surface. With a constant incident light wavelength and a metal species (gold) and thickness, the resonance angle that triggers SPR is dependent on the refractive index of the material near the metal surface. Therefore, a small change in the refractive index of the sensing medium will lead to the occurrence of SPR, which makes it possible for analysis and detection of the refractive index change in the evanescent wave field. Two-dimensional surface plasmon resonance (2D-SPR) can monitor two dimensional and local area changes of the refractive index occurring at the surface of a gold sensor chip. A binding events including mass accumulation cause change of refractive index on the observing plane and positive shift of resonance angle of the area.[9]

1-3 Application of 2D-SPR

In the conventional SPR assay, recognition molecules, which were immobilized on a sensor chip, bind with the target molecules to form a biomolecular complex such as antibody-antigen and probe DNA-target DNA, on a gold chip surface. The refractive index in the evanescent wave field was increased by the biomolecular complex on the surface of gold chip. In addition, the changes of refractive index caused by the binding reaction of biomolecules are monitored by real-time measurements. Thus, SPR sensors have been widely used for determination of affinity constants or concentration measurements of target biomolecules.[10] In these backgrounds, Prof. Hide and Prof. Yanase et al. have monitored the physiological response of RBL-2H3 cells upon antigen stimulation by using SPR sensor for the first time.[15-17] After their

frontier studies, Prof. Shinohara's group used two-dimensional surface plasmon resonance (2D-SPR) measurement to monitor and quantify the allergic response of RBL-2H3 cells at single cell level.[18-20] These results demonstrated that 2D-SPR observation could be used for monitoring the allergic response of IgE-sensitized RBL-2H3 cells upon antigen stimulation as a highly-sensitive, real-time and reagent-less method and might be available for inhibitor screening and its quantification. Prof. Shinohara's group has further considered that 2D-SPR response by the refractive index change at RBL-2H3 cell upon antigen stimulation might be related with the protein kinase C (PKC) translocation to the cell menbrane as Prof Hide's group also suggested.[18] DNP-BSA stimulation on RBL-2H3 cell induced intracellular PKC translocation to the cell membrane and PKC was activated on the membrane. It was expected that the mass change of cell membrane by PKC binding lead the refractive index increase and it could be observed by 2D-SPR image as the local reflection light intensity increase.

1-4 Purpose of this study

Based on the previous our laboratory's studies, I tried to apply 2D-SPR observation for evaluating the anti-allergic effect and anti-cancer effect of natural products. In this study, glycyrrhizic acid (GA) and isoliquiritigenin (ISL) were selected as the typical natural medicines which possessed anti-allergic effect and anti-cancer effect. I expected that GA and ISL could suppress indirectly 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. Besides, inhibition effect on the proliferation of human lung cancer cells was next evaluated by 2D-SPR observation. The refractive index change at cell regions was monitored by 2D-SPR observation and evaluated the anti-proliferation effect of ISL. This study may provide usefulness of 2D-SPR observation for screening anti-allergic effect and for evaluating anti-proliferation effect of natural medicines.

Chapter 2

Materials and Methods

2-1 Materials

RBL-2H3 cell line and A549 cell line were 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). Gibco Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (Tokyo, Japan).Fetal bovine serum (FBS) was obtained from Biowest (Nuaillé, France). Albumin from bovine serum, 2, 4-Dintrophenylated (DNP-BSA) was purchased from Merck Millipore (Massachusetts, USA). Hanks' balanced salts (HBS), 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 pure water. The 50 nm gold thin film-coated high refractive index glass (SF6) chip (18 × 17 mm) was purchased from BAS Ins. (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). The purity of GA and ISL was more than 97%. Penicillin-Streptomycin Solution was purchased from FUJIFILM (Tokyo, Japan). 96 well plate was obtained from CORNING (USA). Culture flask (25 cm^2) was purchased from Thermo Fisher (Waltham, USA).

2-2 Cell culture

RBL-2H3 cells were maintained in EMEM supplemented with FBS (10%), penicillin/streptomycin (1%) in a cell culture flask (25 cm^2) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Before experiments, the RBL-2H3 cells ($1X10^5$ 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). And the cells were incubated in a CO_2 incubator for one day (24 h) to adhere the cells on the chip. Prior to 2D-SPR observation, the culture medium in the cell adhered chip chamber was replaced with HBSS (pH 7.4, 37 °C) and experiments were then conducted under room temperature.

A549 cells were maintained in DMEM supplemented with FBS (10%), penicillin/streptomycin (1%) in a cell culture flask (25 cm^2) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Before experiments, the A549 cells ($1X10^4$ 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). And the A549 cells from other flask was reseeded in each well of a 96-wells plate by $1X10^4$ cells/well. These cells were incubated during 12 hours for cell attachment before the 2D-SPR observation.

2-3 2D-SPR observation system

The 2D-SPR instrument (2D-SPR04A, NTT-AT, Japan) with a collimator lens for parallelizing incident light, a P- and S-changeable polarizer and a cooling CCD camera coupled with four kinds of magnification lens (1X, 2X, 4X and 7X) was used to observe the reflection light image and to monitor the time-course of reflection intensity at the region of interests (Fig. 2-1). The cell attached in a gold chip chamber was set on the prism of the 2D-SPR system with matching oil. The SPR resonance angles at the cell regions were first measured by changing of the incident angle from 49° to 55° at 0.1 steps to determine the SPR angle and the suitable measurement angle for time-course monitoring of the reflection intensity. The measurement angle was determined by the SPR angle -0.5º.

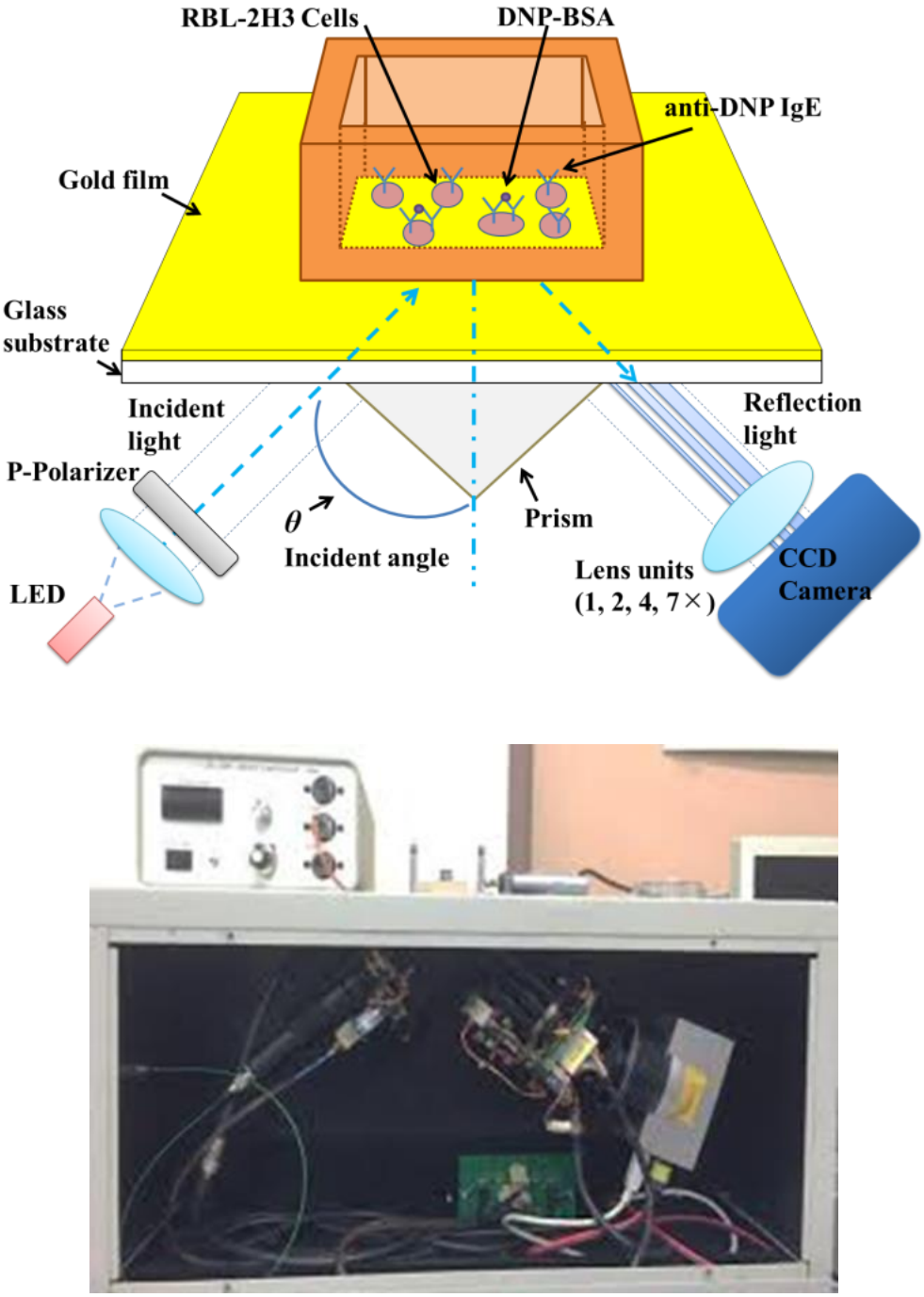

Fig. 2-1 (Upper) The schematic illustration of used 2D-SPR instrument with a collimator lens for parallelizing incident light, a P-polarizer and a cooled CCD camera coupled with four kinds of magnification lens (1X, 2X, 4X and 7X)**. (Lower)** The picture of the used 2D-SPR measurement system.

2-4 The relationship between resonance angle and refractive index

I first investigated the relationship between the refractive index and resonance angle with different concentration of sucrose (from 0.00 to 28.00 % w/w) solution. The refractive index of different concentration of sucrose solution was confirmed by reference (Table 1). The SPR resonance angle against the different concentration of sucrose solution were monitored by the 2D-SPR observation with various incident angles from 49° to 55° every 0.1° (Fig. 2-2). As shown in Fig 2-3, resonance angle was increased as the refractive index increased. From the result, I could easily understand the refractive index of the evanescent field in cells through 2D-SPR observation monitored resonance angle in cell region.

Table 1 The refractive index of sucrose solution in different concentrations.

Concentrantion % w/w	0.00	4.00	8.00	12.00	16.00	20.00	24.00	28.00
n	1.3330	1.3388	1.3448	1.3509	1.3573	1.3639	1.3706	1.3776

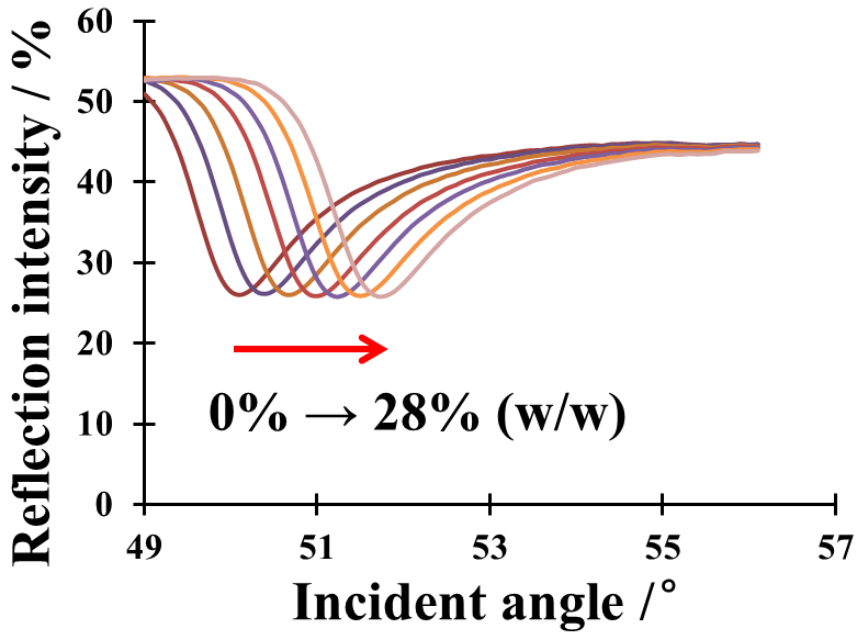

Fig. 2-2 2D-SPR curve against different concentration of sucrose solution. The sucrose concentration increase induced the positive shift of the resonance angle. The concentrations of sucrose were 0.00, 4.00, 8.00, 12.00, 16.00, 20.00, 24.00 and 28.00 % w/w.

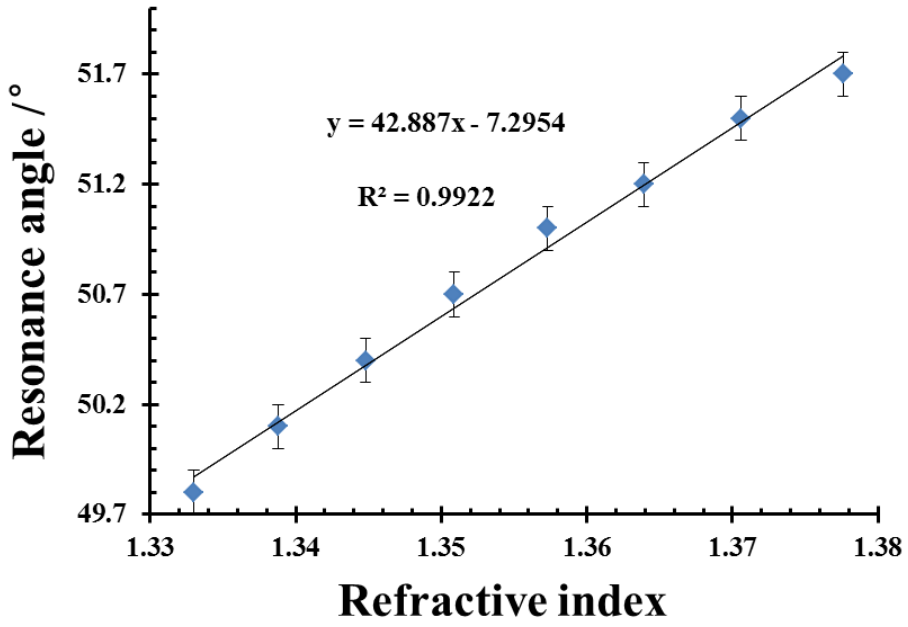

Fig. 2-3 The relationship between resonance angle and refraction index. Resonance angle increased linearly as refractive index increased. The sucrose solution with various refractive index were prepared by dissolving the certain amount of sucrose powder to pure water in the range of 0.00, 4.00, 8.00, 12.00, 16.00, 20.00, 24.00 and 28.00 % w/w.

Chapter 3

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

3-1 Introduction

Some natural medicines have good anti-allergic activity and they have contributed to treat some allergic diseases such as pollinosis, rhinitis, bronchial, asthma dermatitis, and so on.[11] The anti-allergic activity of chemicals including natural medicine could be evaluated usually by several methods such as receptor-based, enzyme-based and cell-based assay.[12-14] But, time consuming, high cost and complex treatment are some common disadvantages in these methods. Thus the more simple, rapid and low cost methods were become necessary and required in screening nature medicines. The rat basophilic leukaemia cell (RBL-2H3) was a wildly known model mast cell used in allergy studies. The degranulation and release of histamine and β-hexosaminidase from RBL-2H3 cells are induced by the antigen stimulation. The mast cell degranulation of released histamine and β-hexosaminidase are usually detected by HPLC analysis and enzymatic activity.

In contract, Hide and Yanase et al. was first time to apply a surface plasmon resonance (SPR) sensor to monitor the RBL-2H3 cell allergic response.[15-17] On the other hand, Prof. Shinohara's group has tried to monitor RBL-2H3 cell response to agonist stimulation by two-dimensional surface plasmon resonance (2D-SPR)

measurement at single cell level.[18-20] The antigen stimulation lead cell response on the IgE-sensitized model mast cell was observed very clearly as expected. These previous studies suggested that 2D-SPR observation was a highly-sensitive, real-time and reagent-less method to monitor the allergic response of RBL-2H3 cells and available for antigen screening and quantification. Prof. Shinohara's group speculated further 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 Prof. Hide et al also considered.[18] It is well known that antigen activates the FcεRIs through the cross-linking of IgE molecules bound on the FcεRIs and the intracellular signaling lead the PKC translocation to trigger the degranulation in mast cells.[21]

In this study, 2D-SPR observation was used to evaluate the anti-allergic effect of natural medicines in mast cell. Glycyrrhizic acid (GA) and isoliquiritigenin (ISL) were selected and tested as the typical natural medicines from Licorice.[22, 23] It has been reported that GA and ISL have inhibition effect on the degranulation in mast cell through the blocking of the Ca^{2+} influx.[24] Another reference data suggested that the suppression of Ca^{2+} concentration suppressed PKC translocation in the mast cell.[25] Based on these references, I consider that GA and ISL could inhibit the PKC (α and β) translocation and the following degranulation after antigen stimulation in RBL-2H3 cells. Thus the suppression of the intracellular reaction might be monitored by 2D-SPR observation rapidly. Therefore, I expected that anti-allergic effect of natural products will be evaluated by 2D-SPR observation.

The chapter 3 has been online published in *Electrochemistry* at October 8, 2020.[39]

3-2 Experimental

3-2.1 2D–SPR observation of cell regions and analysis

The 2D-SPR instrument (2D-SPR04A, NTT-AT, Japan) was used to monitor the time-course of reflection intensity and observe the reflection light (SPR) image at the region of interests. Former to 2D-SPR observation, RBL-2H3 cells were cultured in the cell chip chamber overnight. Then the culture medium was removed and washed with 300 μL HBSS (pH 7.4, 37 °C). After that 300 μL of HBSS with 0.1 μg/mL anti-DNP IgE was injected and incubated for 1 hour to sensitize the RBL-2H3 cells. After the sensitization and washing process, 300 μL of HBSS including 30 μM GA or 50 μM ISL were poured into the cell chip chamber and incubated for 30 min. After the pretreatment of GA and ISL, the solution was replaced with pure HBSS. Then the cell adhered chip was placed on the top of the prism of the 2D-SPR instrument with refractive index matching oil. The 2D-SPR measurement was then conducted in room temperature. At first, the SPR angle was measured at RBL-2H3 cell region and then the measurement angle was set at resonance angle – 0.5° for 2D-SPR observation. Time-course of reflection intensity at each cell regions was monitored following this measurement angle. For the 2D-SPR observation, HBSS (10 μL) was injected at 3 min as the negative control and 10 μL of DNP-BSA solution (10 ng/mL) was injected at 6 min to induce the degranulation of the RBL-2H3 cells. Because of the intracellular reactions, SPR image of RBL-2H3 cell regions became brighter after the

DNP-BSA stimulation. Thus the brightness regions were determined as the cell regions and selected regions of interest was shown with a circle in the 2D-SPR image. In contrast, the darkness region is the gold region. The SPR image of the cell regions were monitored and recorded by CCD camera with the 7× magnification lens.

3-2.2 β-Hexosaminidase assay

Degranulation of RBL-2H3 cells was determined by the enzyme activity of released β-hexosaminidase from the same number of cells. The enzyme activity of β-hexosaminidase was used to evaluate the degranulation inhibition effects of GA and ISL. The β-hexosaminidase released from RBL-2H3 cells which has been pretreatment with GA or ISL and upon same concentration of antigen stimulation.[11] For IgE sensitization, the RBL-2H3 cells were first dispensed into 36 wells in a 96-well plate with EMEM containing 0.45 μg/mL of anti-DNP IgE and incubated for 1 h at 37°C in 5% CO_2. The concentration of RBL-2H3 cells were $5x10^4$ cells/well. The RBL-2H3 cells were washed two times by 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 incubated for 30 min. After the washing process, 10 μL of HBSS containing various concentration of DNP-BSA was added to each well and incubated at 37°C for 10 min to induce the degranulation of RBL-2H3 cells. The experiment was done three times at the same condition. The degranulation reaction was stopped quickly by cooling the well plate in an ice bath for 10 min. Then 30 μL supernatant in each well was 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 M citrate buffer solution, pH 4.5) was added to each well and then incubated at 37°C for 1 hour. The enzymatic reaction was stopped by adding 200 μL of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance of each well solution was measured by 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 measured in the cases with or without pretreatment of the GA or ISL. The degranulation inhibition effect of GA and ISL was evaluated by the released enzyme activity.

3-2.3 Investigation of SPR response mechanism

The experimental procedures of cell culture and 2D-SPR instrument set up are same as described in **3-2.1**. After the sensitization, 300 μL of HBSS contain 10 μM of sphingosine was poured into the cell chip chamber and incubated for 30 min. For the 2D-SPR observation, HBSS (10 μL) was injected at 3 min as a negative control stimulation and 10 μL of DNP-BSA solution (10 ng/mL) was injected at 6 min to induce the degranulation of RBL-2H3 cells.

3-3 Results and discussion

3-3.1 Resonance angle measurement and optimal measurement angle determination for 2D-SPR observation in RBL-2H3 cells

Before using the 2D-SPR observation to evaluate the anti-allergic effect of GA in RBL-2H3 cells, the resonance angle and the optimal measurement were investigated at first. The SPR angle was measured through the monitoring of the reflection intensity at same cell regions and a gold region with a range of incident angles (from 49° to 55° every 0.1°). As show in Fig 3-1, the resonance angle shifted from 51.5° to 52.0° after DNP-BSA stimulation and the maximum change of reflection intensity before and after DNP-BSA stimulation was seen at 50.9°. Therefore, the optimum incident angle was determined at 50.9° and used to monitor the RBL-2H3 cell response by 2D-SPR observation.

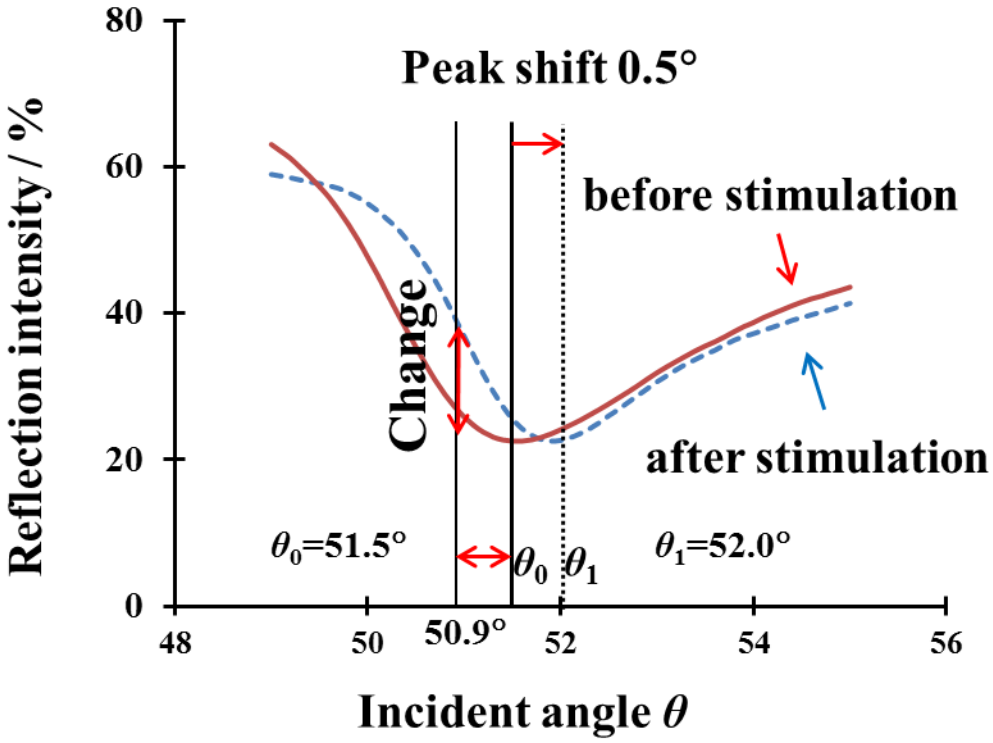

Fig. 3-1 SPR curves at a cell region before and after DNP-BSA stimulation. The measurement angle for 2D-SPR observation was set at initial resonance angle – 0.6° (51.5°).

3-3.2 Evaluation of the anti-allergic effect of GA on RBL-2H3 cell by 2D-SPR measurement

The RBL-2H3 cell response upon DNP-BSA (10 ng/mL) was observed by 2D-SPR instrument. Before the 2D-SPR observation, the culture medium in cell chip chamber was replaced with HBSS. Each cell region of reflection intensity was monitored and recorded by the 2D-SPR instrument at the measurement angle determined. HBSS was injected at 3min as a negative control and DNP-BSA and was injected consecutively at 6 min. As shown in Fig. 3-2C, the reflection intensity was significantly increased after DNP-BSA stimulation at each cell region, but no significant change was observed at a gold region. The time-course measurement demonstrated that the reflection intensity increased slowly after the antigen stimulation and became equilibrium state at 8 min (900 sec.). The SPR images of RBL-2H3 cell regions changed brightly after 10 min from the stimulation as shown in Fig. 3-2A and 3-2B.

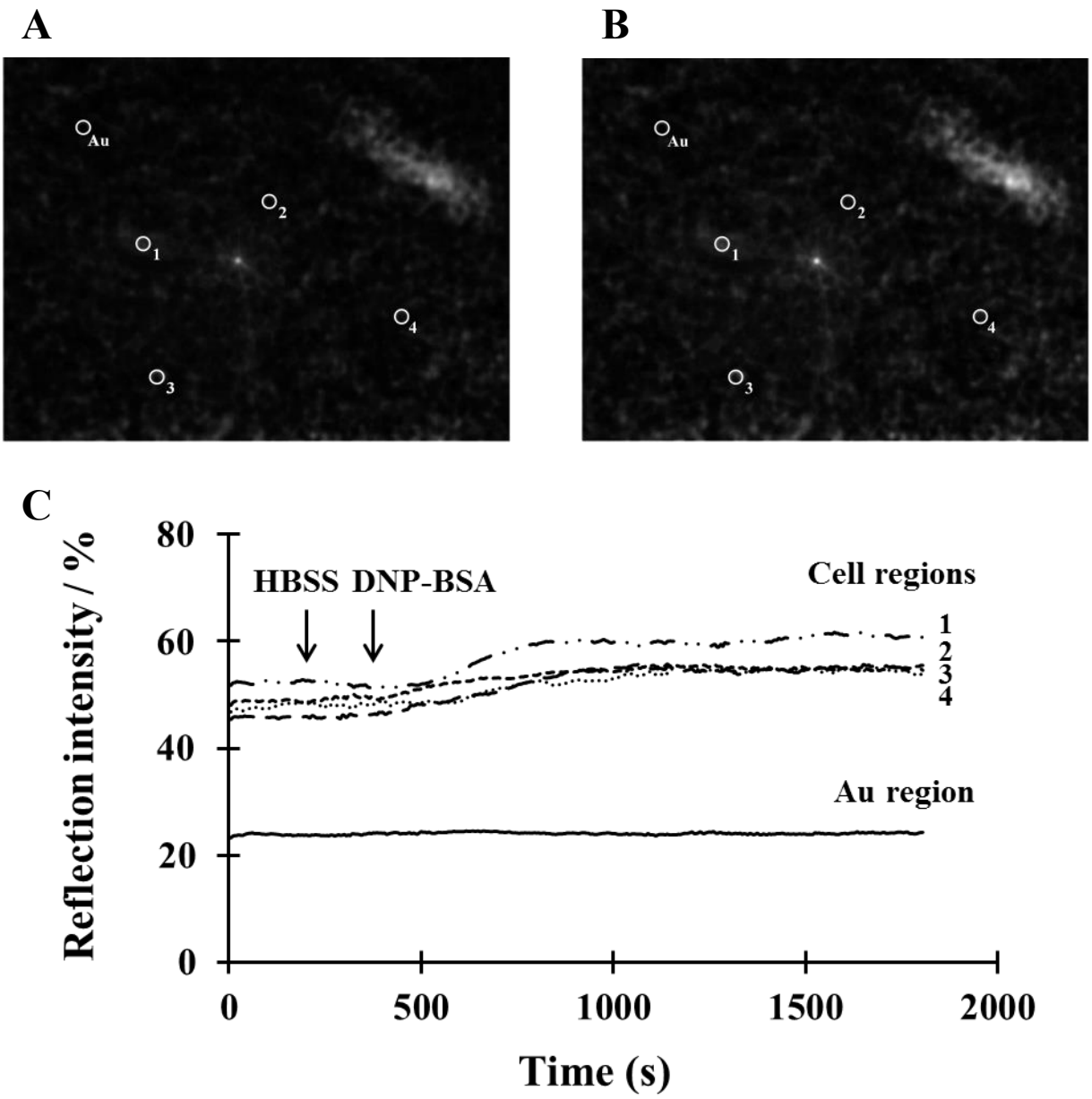

Fig. 3-2 (Upper) SPR images of the RBL-2H3 cell adhered gold chip before (**A**) and after (**B**) addition of 10 ng/mL DNP-BSA without pretreatment of GA. Cell region became clearly bright after 10 min from the stimulation. The SPR image of (**B**) was taken at 10 min after antigen stimulation. (**Lower**) **C:** Time-course of the reflection intensity. The reflection intensity at 4 cell regions increased increased in 2D-SPR observation upon 10 ng/mL DNP-BSA stimulation. 2D-SPR measurement angle was set at 50.9°

To evaluate anti-allergic effect of natural medicines by 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 including 30 μM GA for 30 min. After incubation and washing process, the 2D-SPR observation monitored the RBL-2H3 cell response after antigen stimulation and the reflection intensity data was shown in Fig. 3-3C. The HBSS was added at 3 min as a natural control and DNP-BSA was added at 6 min. The reflection intensity at four cell regions was not significantly changed after the DNP-BSA stimulation. The SPR images showed that a little brightness change of cell regions before and after stimulation as shown in Fig. 3-3A and 3-3B. Prof. Shinohara's previous work[18,19] showed that the reflection intensity increase was obviously involved with the degranulation response of RBL-2H3 cell after DNP-BSA stimulation. In contrast, after the pretreatment with 30 μM GA no change of the reflection intensity was observed upon antigen stimulation. This experimental data was observed suggested that GA has enough inhibition in RBL-2H3 cells to effect the intracellular signaling of degranulation by antigen stimulation.

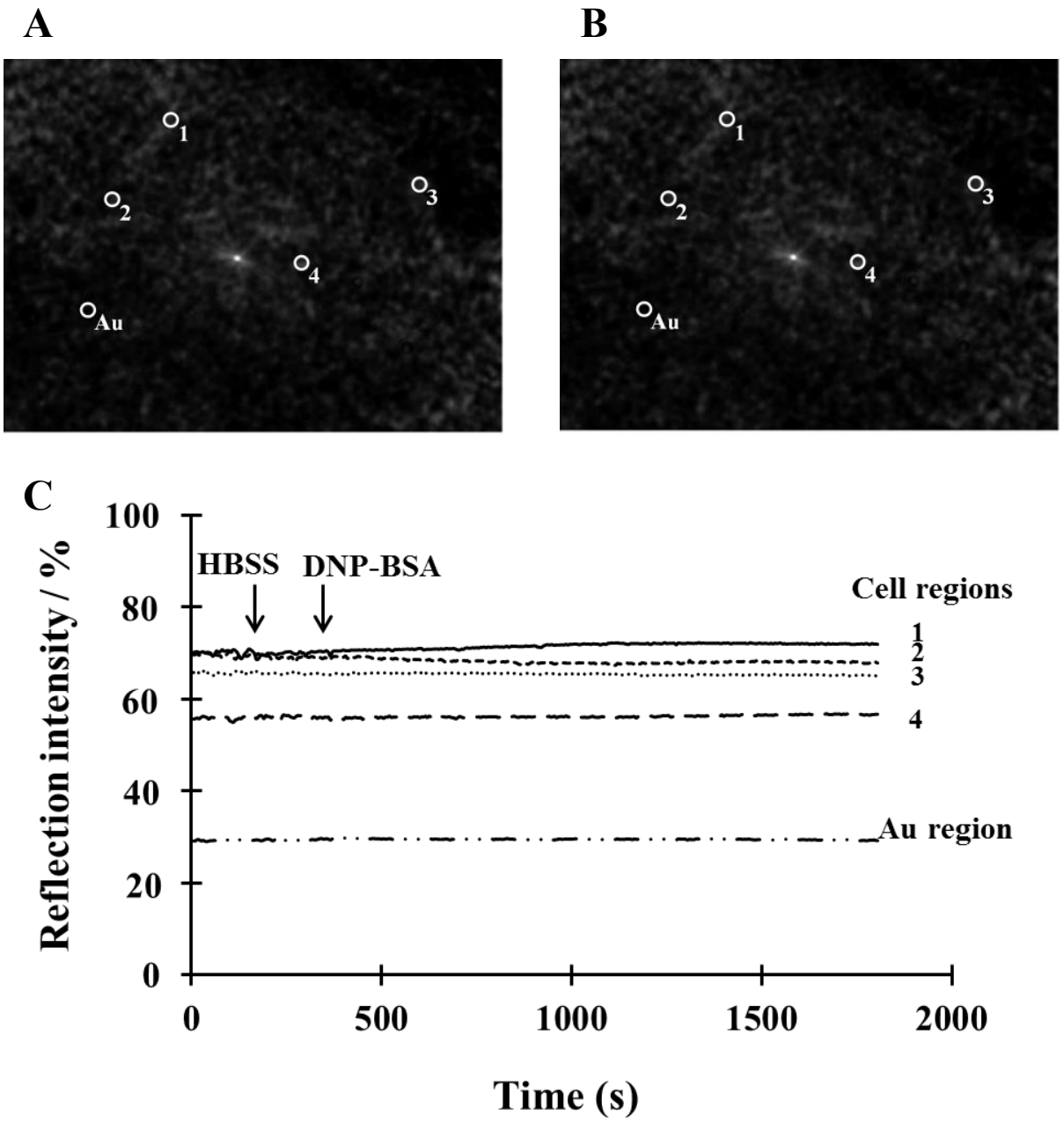

Fig. 3-3 (**Upper**) SPR images of the RBL-2H3 cell adhered gold chip before (**A**) and after (**B**) addition of 10 ng/mL DNP-BSA with pretreatment of GA. The SPR image of (**B**) was taken at 10 min after antigen stimulation. The brightness is almost no change at cell regions. (**Lower**) **C:** Time-course of the reflection intensity at 4 cell regions in 2D-SPR observation upon 10 ng/mL DNP-BSA stimulation with pretreatment of 30 μM GA. 2D-SPR measurement angle was set at 50.9°.

The inhibition effect of GA on RBL-2H3 cell allergic response was examined in the concentration range of lower than 30 μM by reflection intensity change at the cell regions. The time-course of reflection intensity change upon the antigen stimulation after the pretreatment after the pretreatment with different concentration of GA solution was shown in Fig. 3-4. The reflection intensity average at five cell regions was GA-concentration as shown in Fig. 3-5A. The IC_{50} of GA was estimated to be 2 μM.

On the other hand, GA-concentration dependent of the relative enzyme activity of β-hexosaminidase released from RBL-2H3 cells by the DNP-BSA stimulation was shown in Fig. 3-5B. The experiment was done three times at the same condition. The IC_{50} of GA was estimated to be 6 μM by the β-hexosaminidase assay. The 2D-SPR evaluation data and β-hexosaminidase assay data were suggested that 2D-SPR observation was applicable as same as β-hexosaminidase assay for evaluating anti-allergic effect of GA.

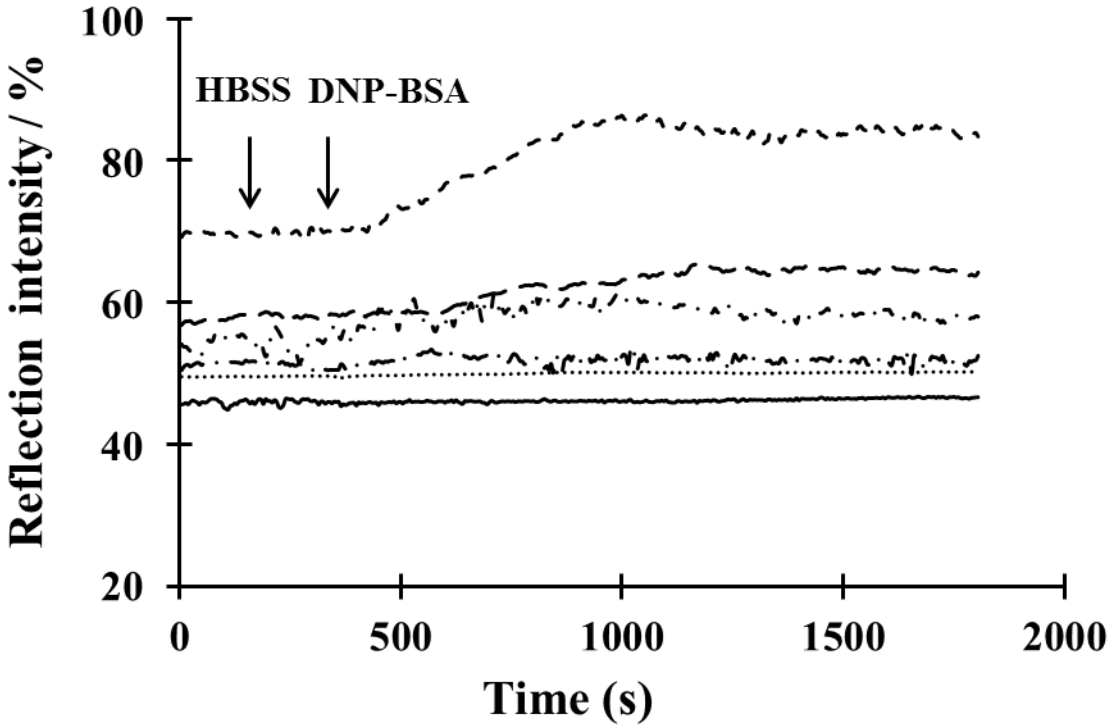

Fig. 3-4 The time-course change of the reflection intensity at a cell region upon 10 ng/mL DNP-BSA stimulation after pretreatment with various concentration of GA was obtained. The GA concentration of pretreatment were 0 μM (– – –), 1 μM (— —), 5 μM (–·–·–), 10 μM (–··–), 20μM (·····), 30μM (——).

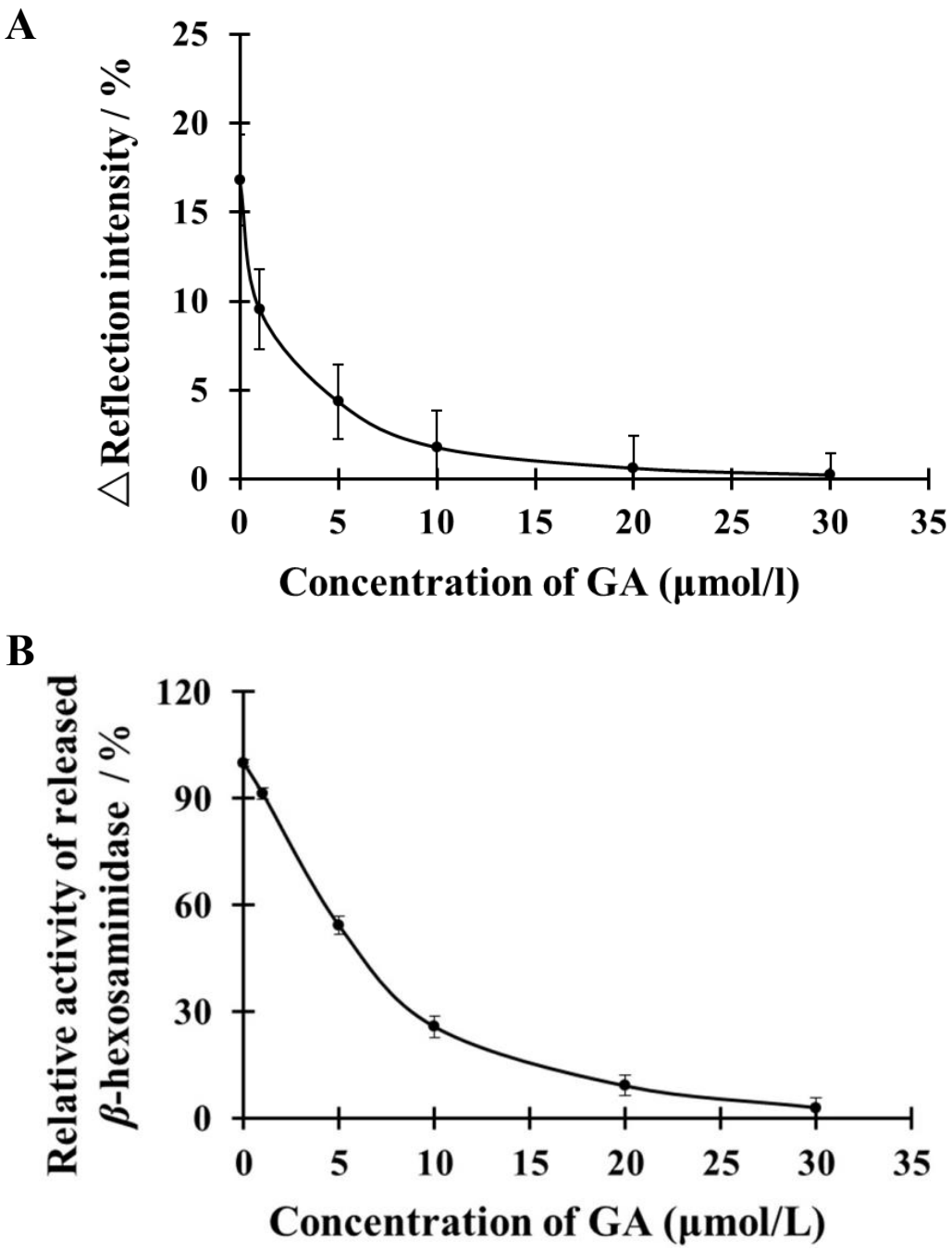

Fig. 3-5 A: Dependence of the reflection intensity increase average at 5 cell regions. on the concentration of GA in the pretreatment. B**:** Dependence of the relative activity of β-hexosaminidase released from cells upon 10 ng/mL DNP-BSA stimulation on the concentration of GA in the pretreatment. The results are expressed as the mean ± SD from three experiments.

3-3.3 Evaluation of the anti-allergic effect of ISL on RBL-2H3 cell by 2D-SPR measurement

Isoliquiritigenin (ISL) as another degranulation inhibitor was evaluated by 2D-SPR measurement. The experimental process was same as described in 3-2. In the absence of ISL pretreatment, the reflection intensity was significant increased after DNP-BSA was injected at 6 min as shown in Fig. 3-6C. The SPR image of RBL-2H3 cell regions changed brightly after 10 min from the stimulation as shown in Fig. 3-6A and 3-6B. In contrast, after 30 min pretreatment with 50 μM of ISL, the reflection intensity at four cell regions showed almost no change upon the DNP-BSA stimulation as shown in Fig.3-7C. In the SPR image, the brightness of cell regions changed a little before and after DNP-BSA stimulation as shown in Fig. 3-7A and 3-7B. These data suggested that 2D-SPR measurement successfully achieved to monitor the inhibition effect of ISL on the degranulation reactions in RBL-2H3 cells.

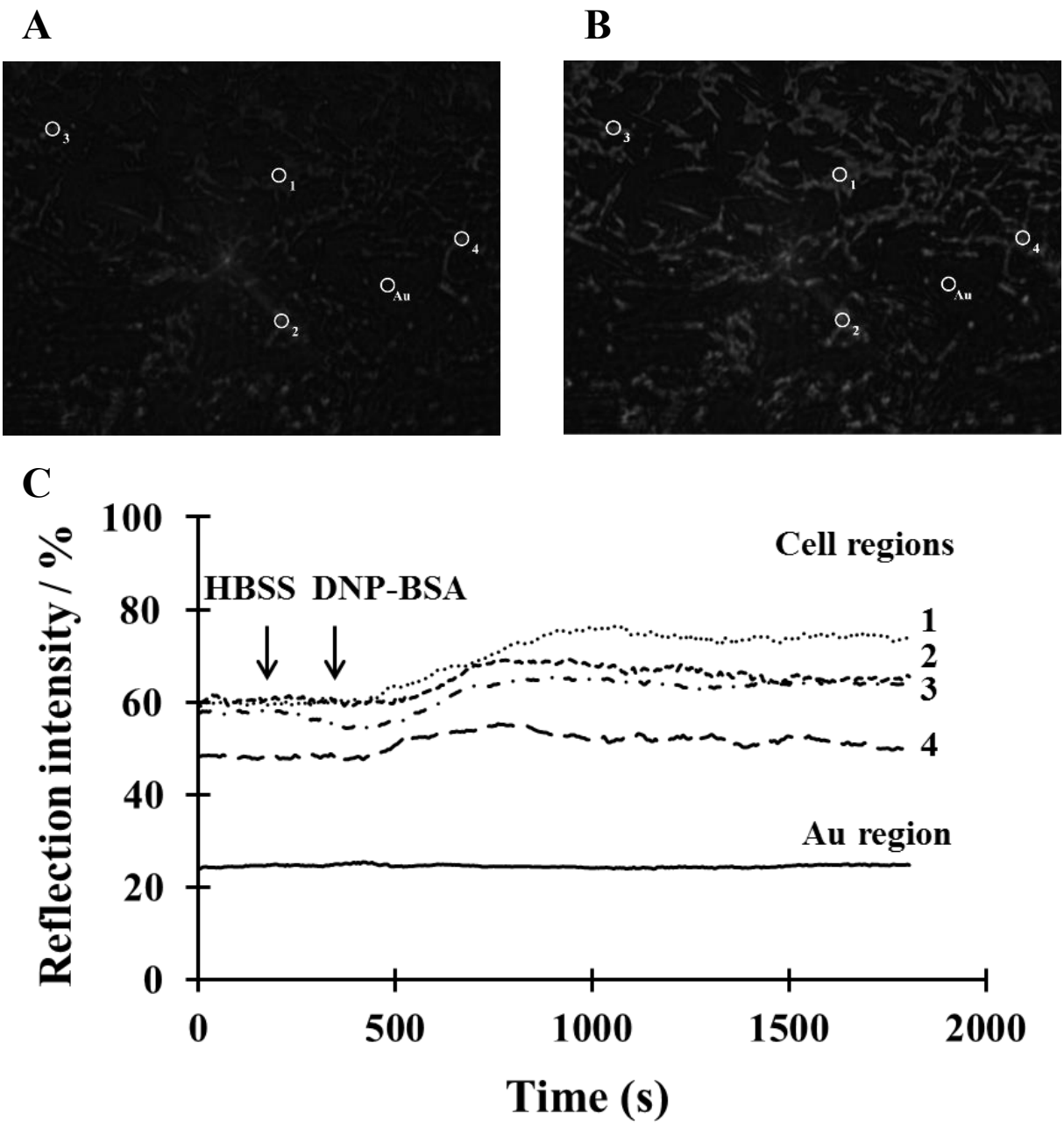

Fig. 3-6 (Upper) SPR images of the RBL-2H3 cell adhered gold chip before (**A**) and after (**B**) addition of 10 ng/mL DNP-BSA without pretreatment of ISL. Cell region became clearly bright after 10 min from the stimulation. **(Lower) C:** Time-course of the reflection intensity increase at 4 cell regions increased in 2D-SPR observation upon 10 ng/mL DNP-BSA stimulation. 2D-SPR measurement angle was set at 50.9°.

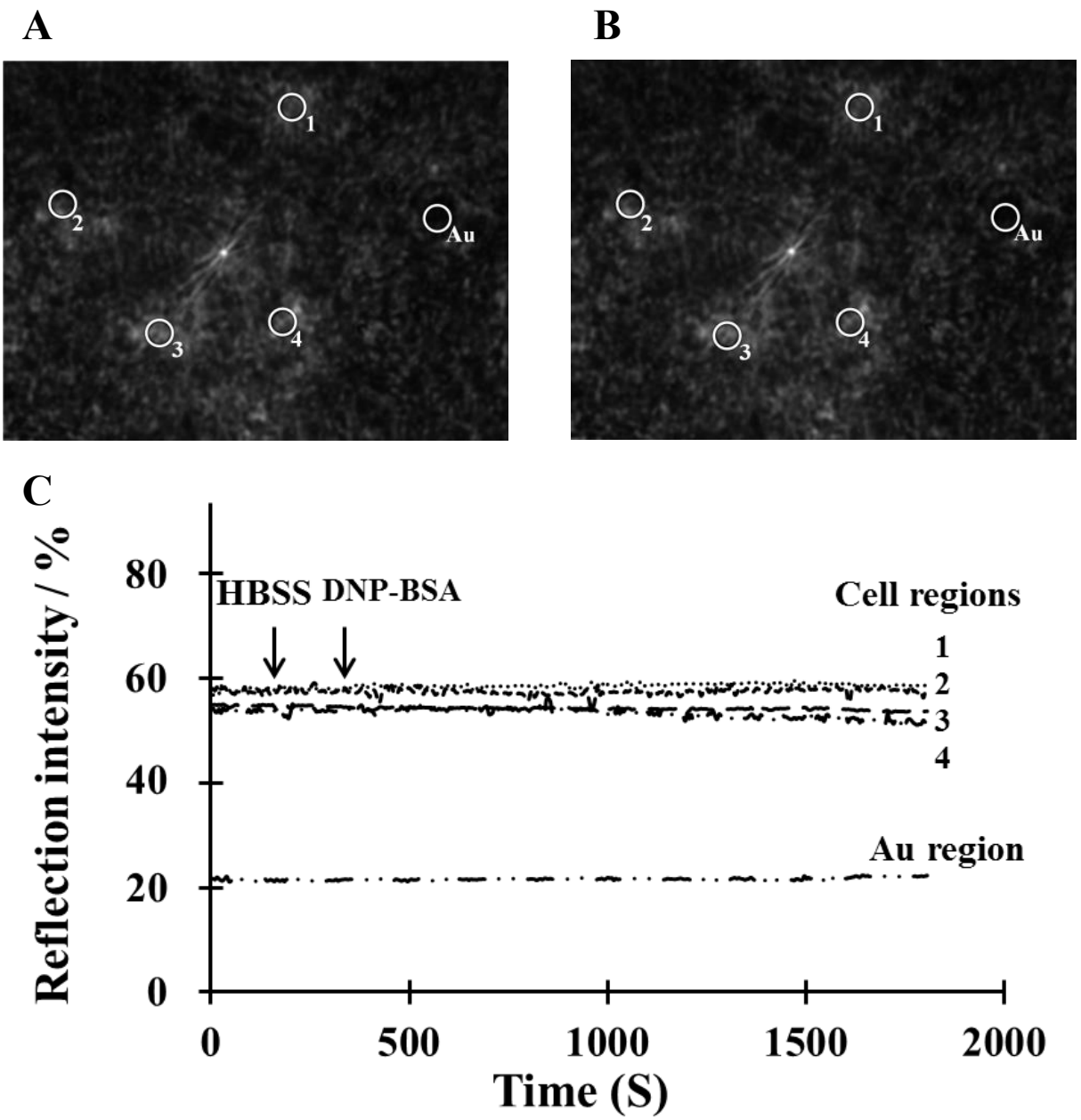

Fig. 3-7 **(Upper)** SPR images of the RBL-2H3 cell adhered gold chip before (**A**) and after (**B**) addition of 10 ng/mL DNP-BSA with pretreatment of ISL. The SPR image of (**B**) was taken at 10 min after antigen stimulation. The brightness was almost no change at cell regions. **(Lower) C:** Time-course of the reflection intensity at 4 cell regions showed little change in 2D-SPR observation upon 10 ng/mL DNP-BSA stimulation after pretreatment of 50 μM ISL. 2D-SPR measurement angle was set at 50.9°.

The ISL-concentration dependence of the suppression of reflection intensity was demonstrated in 2D-SPR measurement. It was shown in Fig. 3-8. ISL was tested as an inhibitor in the concentration range lower than 50 μM as shown in Fig. 3-9A. The IC_{50} of ISL was estimated to be 8 μM. On the other hand, ISL-concentration dependence of the relative activity of β-hexosaminidase released from RBL-2H3 cells upon DNP-BSA stimulation was shown in Fig. 3-9B. The experiment at the same condition was done three times. The IC_{50} of ISL was estimated to be 14 μM by this β-hexosaminidase assay. The slight larger IC_{50} of ISL was reported by another researchers.[23] Based on these data, I demonstrated that the 2D-SPR observation could successfully evaluate the degranulation inhibition effect of ISL too.

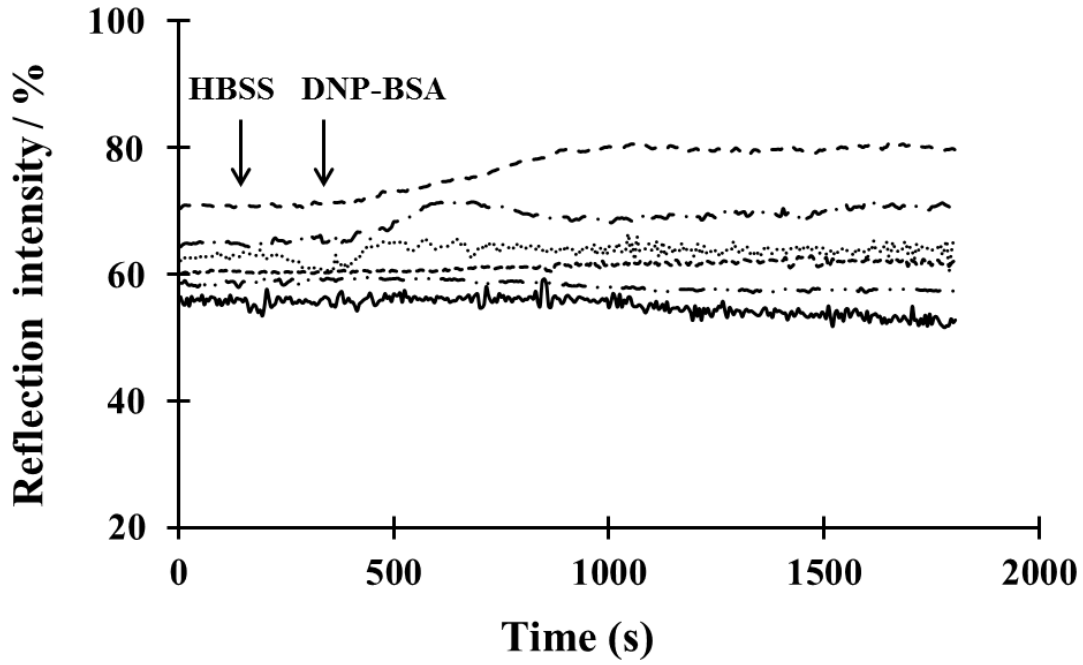

Fig. 3-8: The change of time-course of reflection intensity at one cell region upon 10 ng/mL DNP-BSA stimulation after pretreatment with various concentration of ISL. The ISL concentration of pretreatment were 0 μM (— —), 5 μM (−·−·−), 10 μM (·····), 20 μM (– – –), 30 μM (−··−), 50 μM (——).

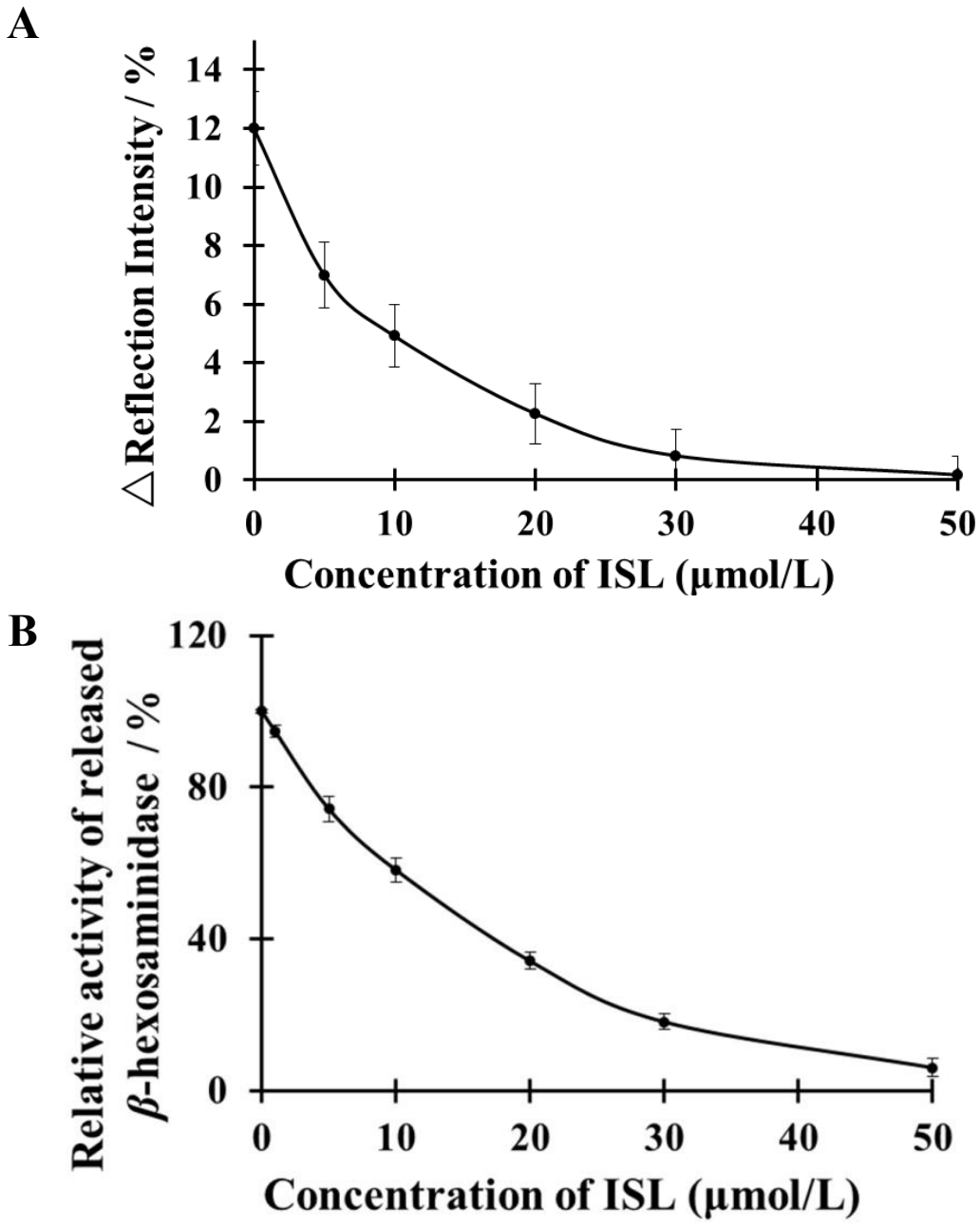

Fig. 3-9 A: ISL concentration dependence of reflection intensity increase at 5 cell regions upon DNP-BSA stimulation. **B:** ISL concentration dependence of the relative activity of β-hexosaminidase released from cells upon 10 ng/mL DNP-BSA stimulation. The results are expressed as the mean ± SD from three experiments.

3-3.4 Investigation of SPR response mechanism

The 2D-SPR observation is not only sensitive but also able to monitor a little change of refractive index in the evanescent field (less than the wavelength of incident light) at cell bottom. Prof. Shinohara and I have hypothesized that the reflection intensity change might be related with the PKC (α and β) translocation in RBL-2H3 cells as previously reported by Hide et al.[18] To understand the SPR response mechanism, 10 μM sphingosine as a PKC specific inhibitor[16] was exposed to RBL-2H3 cells for 30 min in pretreatment process before DNP-BSA stimulation. The reflection intensity change was monitored by the 2D-SPR instrument. The SPR response was significantly suppressed with the pretreatment of sphingosine, as I expected and shown in Fig. 3-10. This result indicated that PKC translocation certainly contributed to the reflection intensity increase in 2D-SPR observation. Even though the detail mechanism of inhibition effect by GA and ISL against PKC translocation was still unclear, our results and reference data[24-27] suggested that GA and ISL might inhibit the upstream of PKC translocation inducing the degranulation.

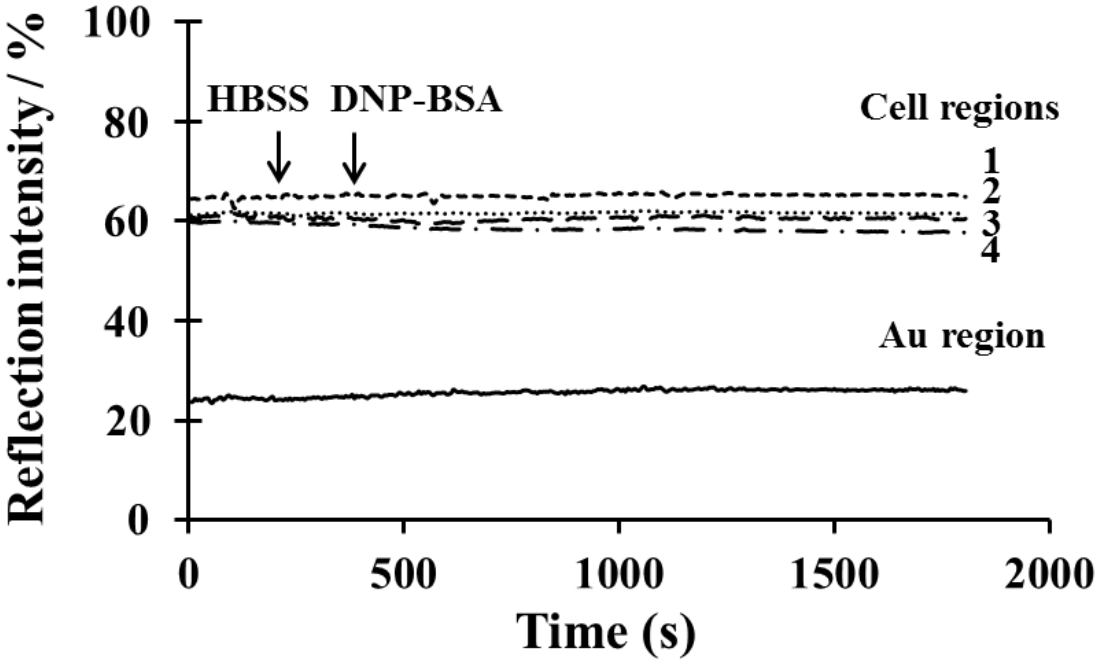

Fig. 3-10 Time-course of the reflection intensity upon 10 ng/mL DNP-BSA stimulation with the pretreatment of 10 μM sphingosine at 4 cell regions. The reflection intensity at individual cell regions did not increase after DNP-BSA stimulation with the sphingosine pretreatment for 30 min.

3-4 Conclusion

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

Chapter 4

Evaluation of the Anti-Proliferation Effect of Natural Medicine on Lung Cancer Cells by Using Two-Dimensional Surface Plasmon Resonance Observation

4-1 Introduction

Lung cancer is one of the most common tumors and leads over 1 million persons death each year all over the world. The non-small cell lung carcinoma (NSCLC) accounts for approximately 75-85% of lung cancers.[28] In recent years, lung cancer mortality has been increasing because of the smoking habit and air pollution. The poor survival for lung cancer is due to lack of effect therapy. Therefore, it could be necessary for screening various of novel compounds that have the anti-cancer effect to treat lung cancers.

Isoliquiritigenin (ISL), a flavonoid ioslated from licorice, has various biochemical activities, such as anti-oxidant, anti-inflammatory and anti-cancer effects.[29-31] ISL exhibits an inhibitory effect on proliferative activity in prostate cancer, hepatocellular carcinoma, breast cancer, melanoma and lung cancer cells. ISL further induces apoptosis and autophagy in breast cancer and endometrial cancer cells.[32-37] In recent years, the anti-cancer mechanism of ISL has been widely studied. Previous studies suggested that ISL induced cell cycle arrest in the G2/M phase through the p53/p21

pathway and promoted cell apoptosis and autophagy through activation of the extracellular signal-regulated kinase pathway.[37] On the other hand, ISL is also involved in phosphatidylinositol 3-kinase (PI3K) /AKT serine/ threonine kinase (AKT) signaling pathway to induce cell apoptosis and proliferation.[38]

There are some methods for screening the anti-cancer effect of natural compounds such as cell counting assay, trans-well invasion assay, western blot analysis and flow cytometric assay. In the previous study, Prof. Shinohara's group including me has tried to monitor and quantify the allergic response of RBL-2H3 cells upon antigen stimulation by two-dimensional surface plasmon resonance (2D-SPR) measurement at single cell level.[18, 19] And I have successfully achieved to evaluate the anti-allergic effect of traditional natural medicines (glycyrrhizic acid (GA) and isoliquiritigenin (ISL)) by using a 2D-SPR observation in quick and simple manner as described in Chapter 3. This previous study suggested that 2D-SPR method might be promising to screen anti-allergic components of natural products and useful to discuss the inhibition mechanism in individual cells.

Based on this background, I further aimed to evaluate the anti-cancer effect of natural product by using 2D-SPR observation in this study. I tried to apply 2D-SPR observation to evaluate the anti-proliferation effect of ISL on a model lung cancer cell.

4-2 Methods

4-2.1 Cell culture

In this study, the A549 cell line from adenocarcinomic human alveolar basal epithelial cell was used as a model of non-small cell lung cancer cell. A549 cells were maintained in DMEM supplemented with FBS (10%), penicillin/streptomycin (1%) in a cell culture flask (25 cm^2) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Before experiments, the A549 cells ($1x10^4$ cells in 300 μL) were reseeded on a 50 nm thick gold thin film-coated high refractive index glass (SF6) chip with a rectangular well of flexiPERM (11 × 7 × 10 mm). A549 cells were reseeded to a 96-wells plate too and incubated for 12 hours in order to adhere the cells.

4-2.2 Microscopic observation of cell growth

A549 cells were seeded into a 96-well plate ($1x10^4$ cells/well). 20μL of ISL medium was added in each well at various concentrations (20 μM, 40 μM, 60 μM and 80 μM) after cell attachment. 20 μM of medicine including 0.1% DMSO was used as the negative control. The number of viable cell was scored by microscopic observation after 12 h, 24 h, 36 h, 48 h and 72 h, respectively.

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4-2.3 2D–SPR observation of cell proliferation

The 2D-SPR instrument (2D-SPR04A, NTT-AT, Japan) with a collimator lens for parallelizing incident light and a P- and S-changeable polarizer and a cooling CCD camera coupled with four kinds of magnification lens (1X, 2X, 4X and 7X) was used

to take the reflection light image and to measure the SPR curve and to monitor the time-course of reflection intensity at the region of interests. After A549 cells attached on a gold chip, 20 μL of ISL (20, 40, 60, 80 μM) or 0.1% DMSO including medium was added into a cell chip chamber and then incubate at 37 °C. After 0 h, 12 h, 24 h, 36 h, 48h and 72 h respectively, the cell chip was placed on the top of the prism of the 2D-SPR instrument and the 2D-SPR measurement was conducted under room temperature. SPR curve was measured at a cell region by incident angle change from 49° to 55° with 0.1° resolution. The SPR image of the A549 cell regions were monitored and recorded by using CCD camera through the 4× magnification lens.

4-3 Results and discussion

4-3.1 2D-SPR observation for monitoring of RBL-2H3 cell proliferation

To evaluate the anti-proliferation effect of ISL, I tried to monitor RBL-2H3 cell proliferation by 2D-SPR observation at first. The SPR angle was first measured to monitor the refractive index change by reflection intensity change at same cell regions through changing the incident angles from 49° to 55° every 0.1°. Images of cell region were obtained at every 1 h, 4 h, 12 h, 24 h and 48 h from the seeding by recoding with a CCD camera as shown in Fig. 4-1. As shown in Fig. 4-1, RBL-2H3 cells proliferated on the gold sensor chip normally. And the SPR angle curves at the same cell region were measured at each time and were shown in Fig. 4-2A. The

resonance angle was shifted positively by the RBL-2H3 cell proliferation (Fig. 4-2B). These data suggested that RBL-2H3 cell proliferation was successfully observed and monitored by 2D-SPR observation. It my be useful in evaluating the anti-proliferation effect of ISL on A549 cells.

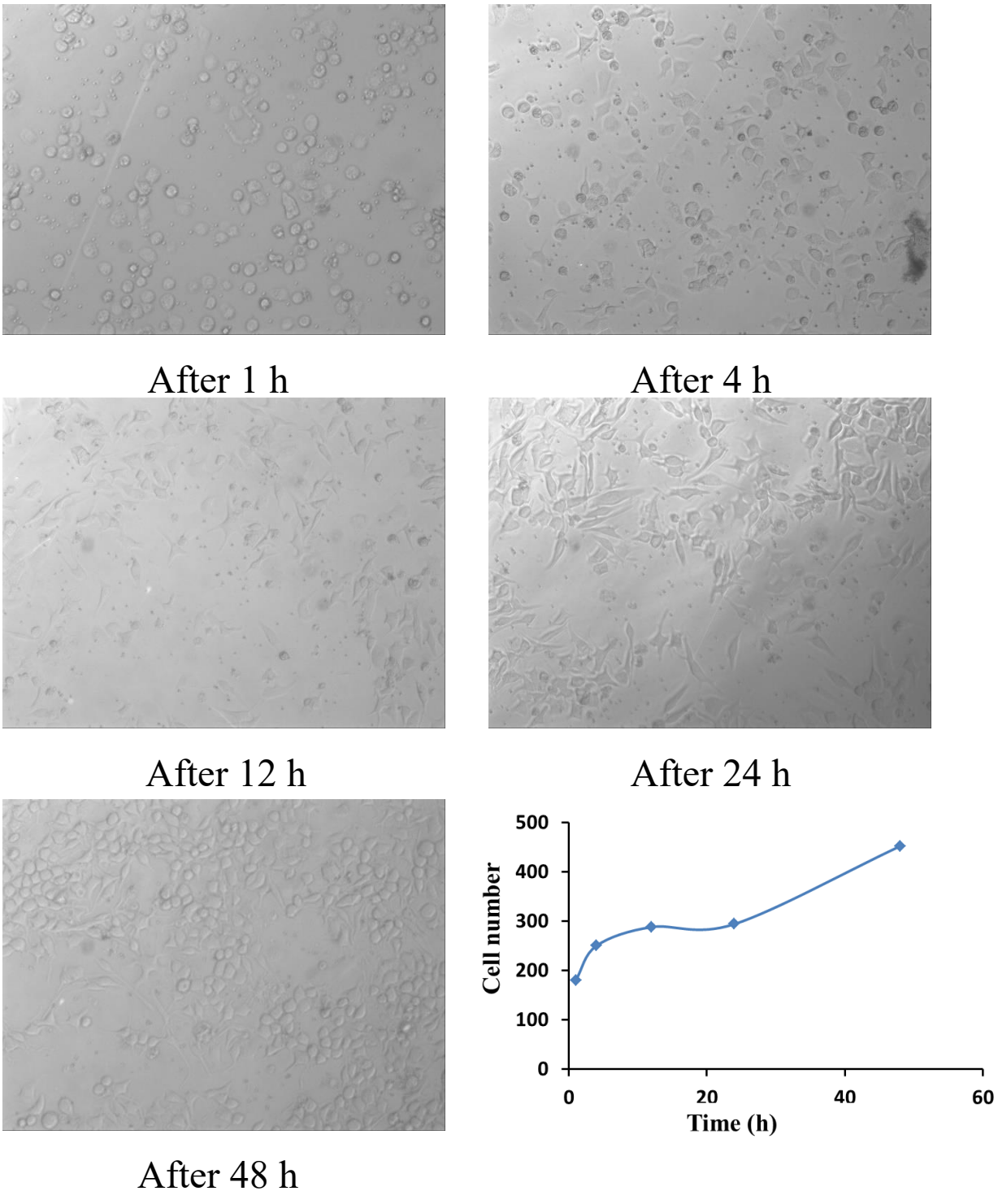


Fig. 4-1 SPR images of RBL-2H3 cell proliferation at each incubation time.

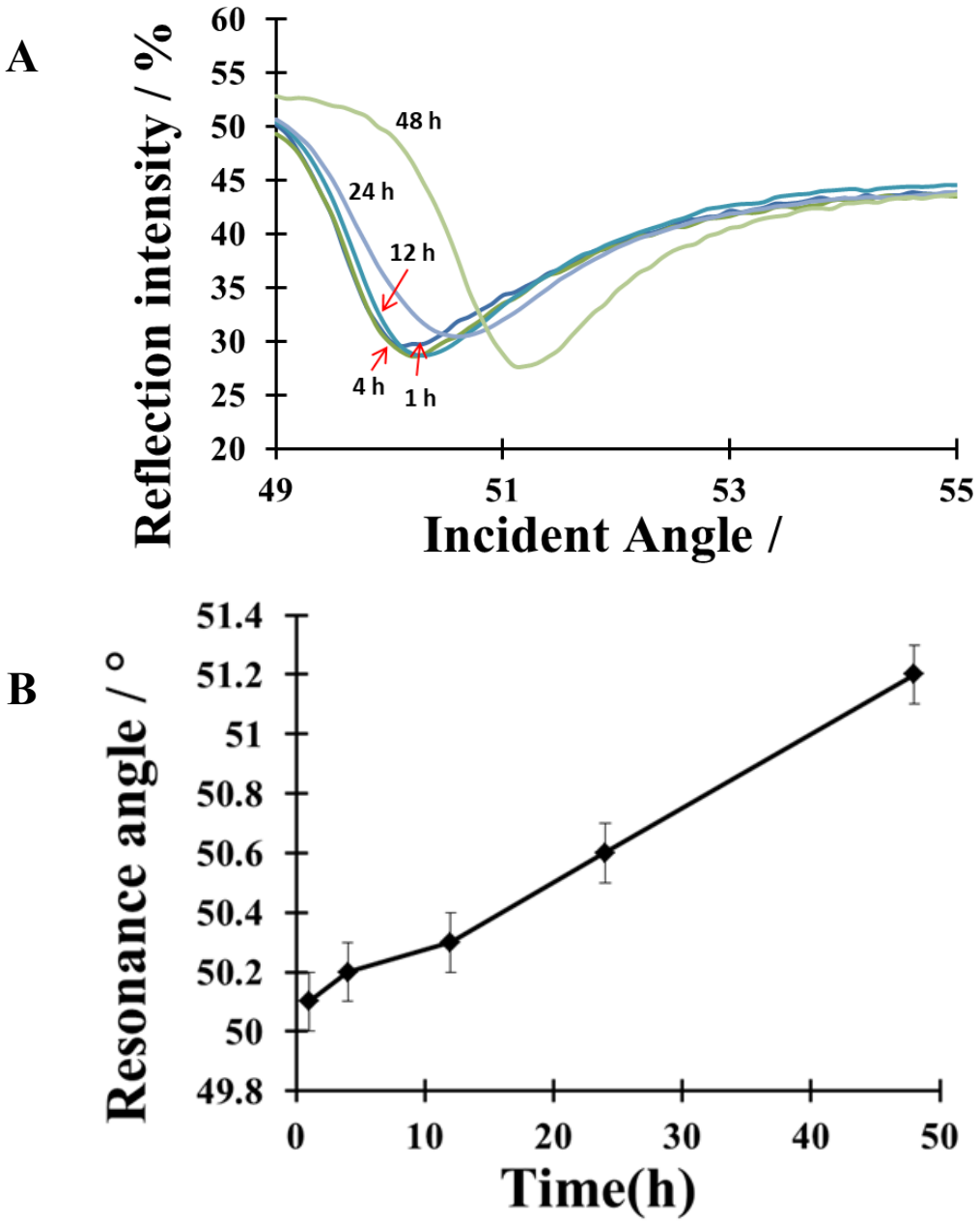


Fig. 4-2 A: Shift of SPR curves of a wide cell region by incubation time. It was depended on the RBL-2H3 cell proliferation. **B:** The resonance angle of the cell region increased by the RBL-2H3 cell proliferation.

4-3.2 Inhibition effects of ISL on A549 cell proliferation

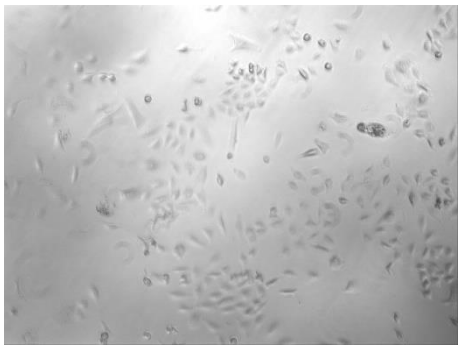
I evaluated the inhibition effect of ISL on the cell growth of the human lung cancer cell line A549. The treatment of A549 cells with various concentration of ISL was done in a time-dependent manner. The A549 cells were treated with 100 μM ISL or 0.1% DMSO including medium for 0 h, 12 h, 24 h, 36 h and 48h in a 96 well plate. The microscopic image pictures were taken by a CCD camera as shown in Fig. 4-3A and 4-3B. The A549 cells growth curve suggested that the A549 cell proliferation was obviously suppressed in the presence of 100 μM ISL as shown in Fig. 4-3C.

A

NC group

0 h

24 h

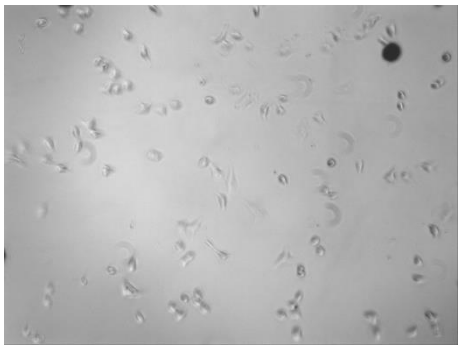


36 h

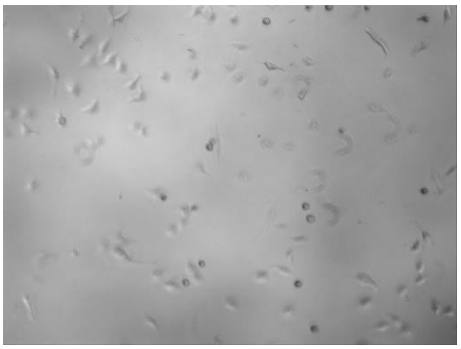
48 h

B

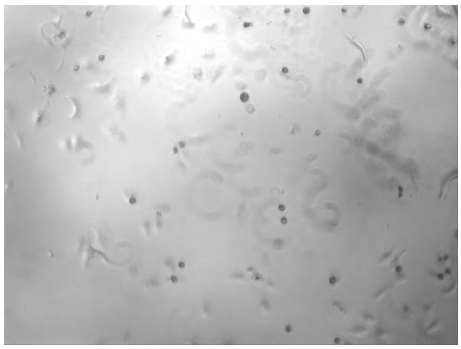
100 μM ISL



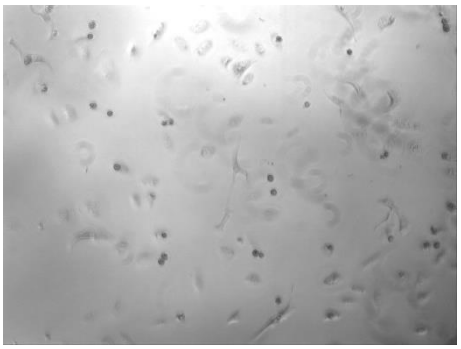
0 h



24 h



36 h



48 h

C

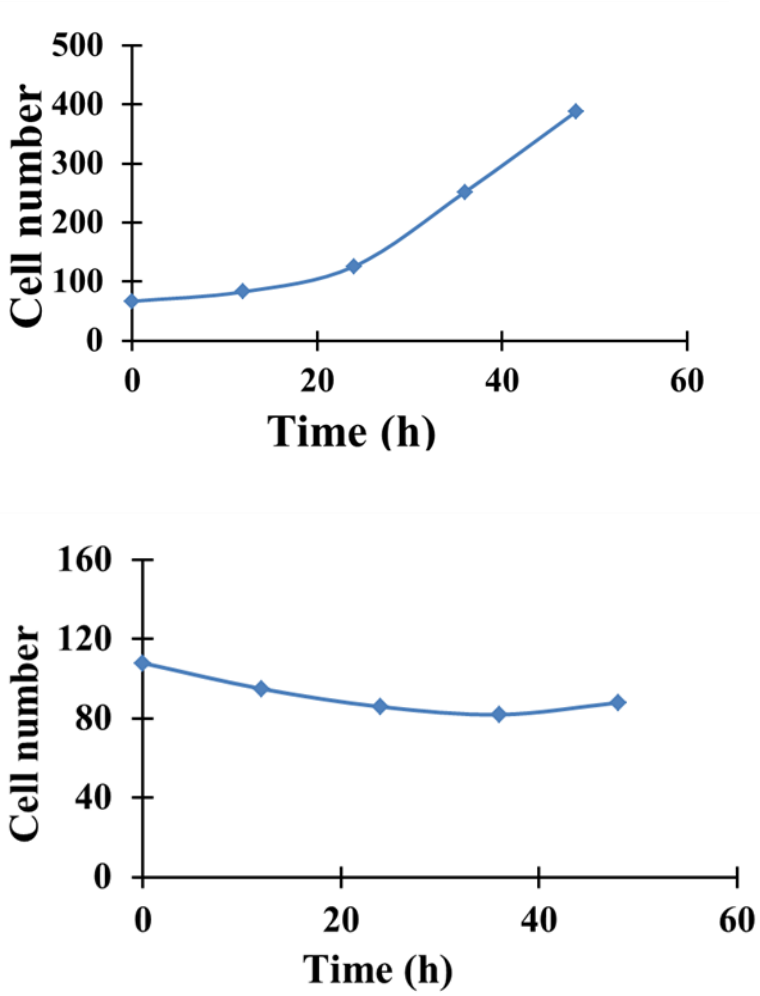


Fig. 4-3 A: Microscopic observation of of A549 cell growth without treatment of ISL at 0 h, 24 h, 36 h and 48 h. **B:** Microscopic observation of the A549 cell growth in wells with treatment of 100 μM ISL at 0 h, 24 h, 36 h and 48 h. **C:** Upper is the A549 cells growth curve in the absence of ISL and lower is the A549 cells growth curve in the presence of 100 μM ISL.

4-3.3 2D-SPR observation for evaluation of the anti-proliferation effect of ISL on A549 cells

To evaluate the anti-proliferation effect of ISL on A549 cells by continuous 2D-SPR observation, The SPR angle was first measured at wide cell region by incident angles from 49° to 55° every 0.1°. The SPR image of A549 cells on the same chip with and without treatment of 100 μM ISL on various times was shown in Fig. 4-4A and 4-4B. The A549 cells increased significantly for 48 hours in the absence of ISL but increased slowly in the presence of 100 μM ISL. The SPR curves of a A549 cell wide region change without and with treatment of ISL were shown in Fig. 4-5A and 4-5B. By comparison of SPR images (Fig. 4-4A and 4-4B), it was shown that the proliferation of A549 cells became slow by the 100 μM ISL treatment This proliferation suppression effect by ISL might be considered as the inhibitory effect on the some enzymes concerned with proliferation signaling pathway as described in the Chapter introduction (section 4-1). The A549 cell proliferation induced the positive change of resonance angle by 2D-SPR observation without ISL treatment. On the other hand, in the presence of 100 μM ISL the resonance angel was almost no changed. It was suggested that the 2D-SPR observation could evaluate the anti-proliferation effect of ISL. Besides, 2D-SPR observation monitored the proliferation of A549 cells in a single cell ROI was also achieved as shown in Fig. 4-6A and 4-6B. In the absence of ISL (control group), the time-course of the resonance angle of single A549 cell ROIs showed different pattern. However, the time-course of resonance angle of each single A549 cell showed almost same pattern

as negative shift after 12 hours in the presence of ISL.

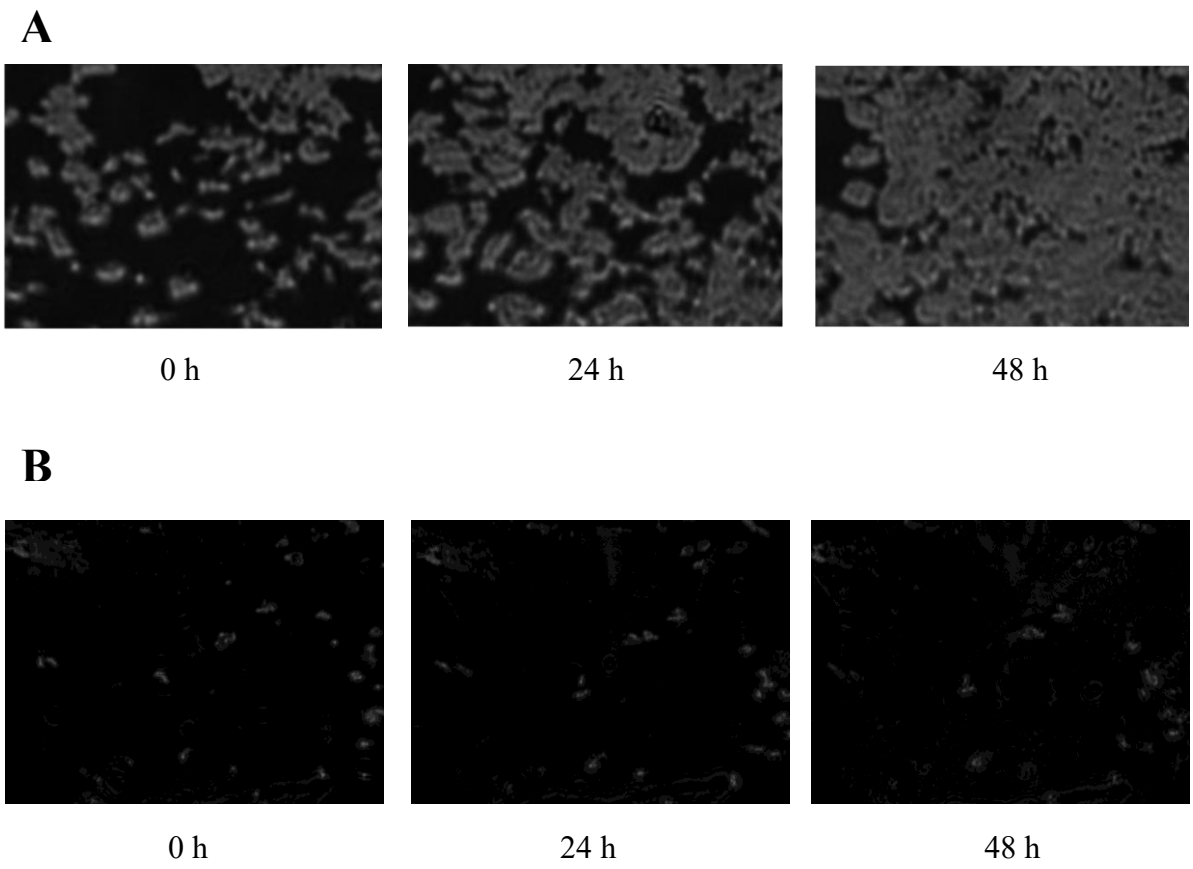


Fig. 4-4 SPR images of A549 cells in the various incubation time. **A:** the SPR image of A549 cells without treatment of ISL. **B:** the SPR image of A549 cells with treatment of ISL. The A549 cell growth without ISL treatment is faster than the cells growth with ISL treatment.

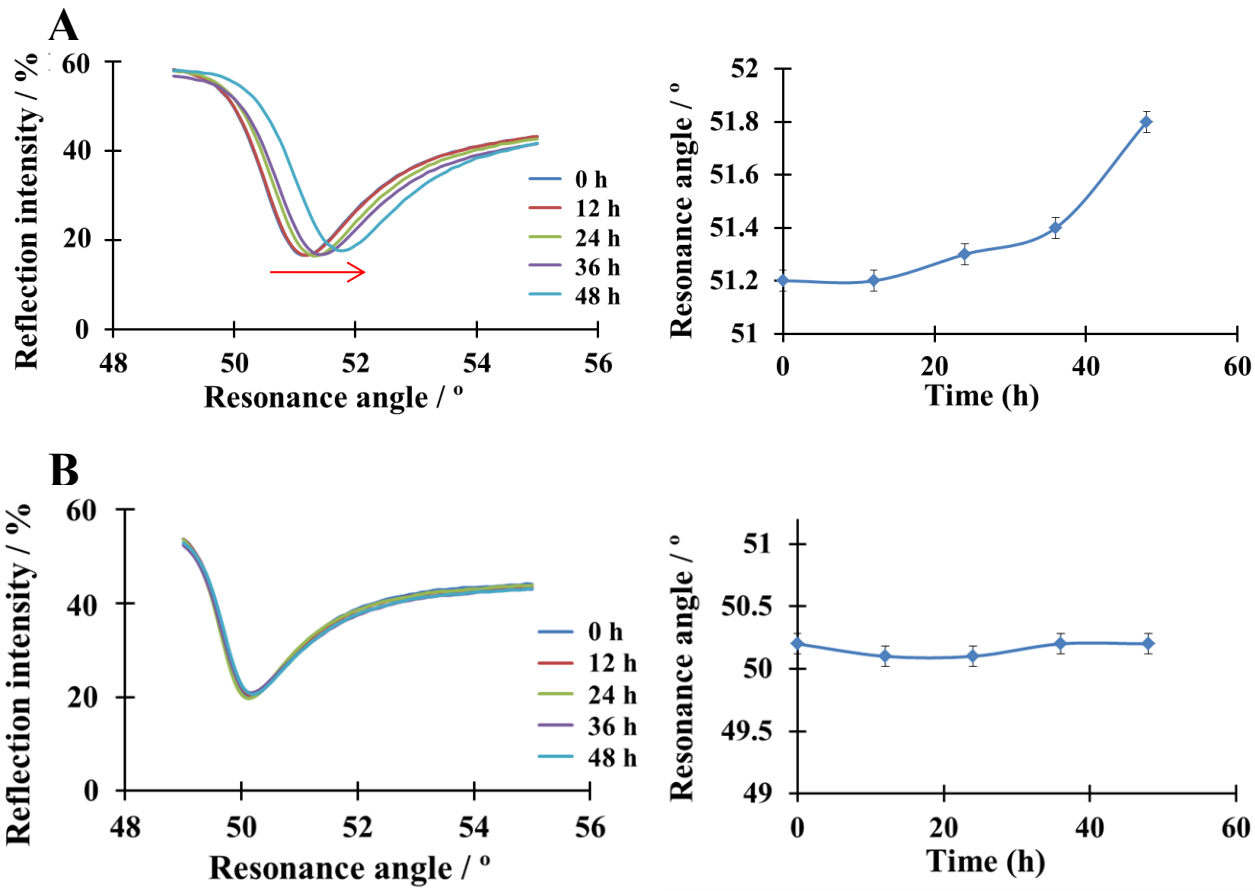
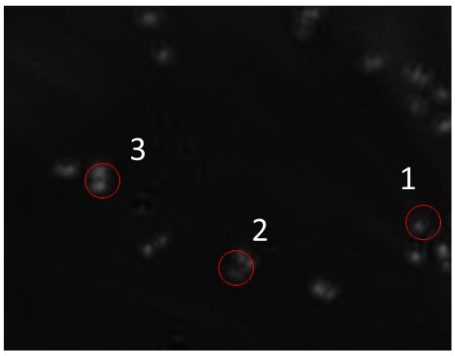
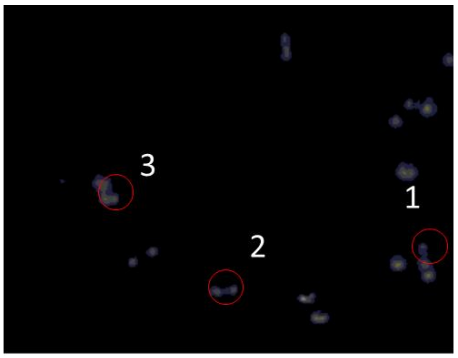


Fig. 4-5 A-1 and **B-1** show the SPR curves on the wide ROI of A549 cell at various incubation time. **A-2** and **B-2** show the SPR angle change against the incubation time.

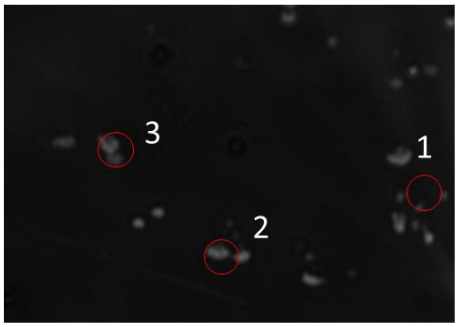
A



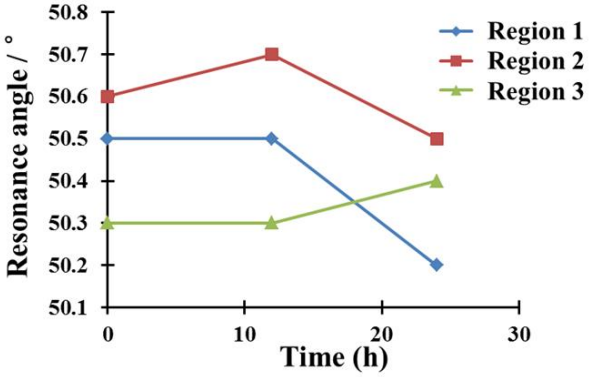
(-) ISL A549 cell region at 0 h



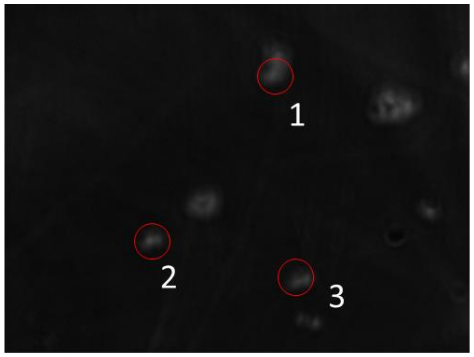
(-) ISL A549 cell region at 12 h



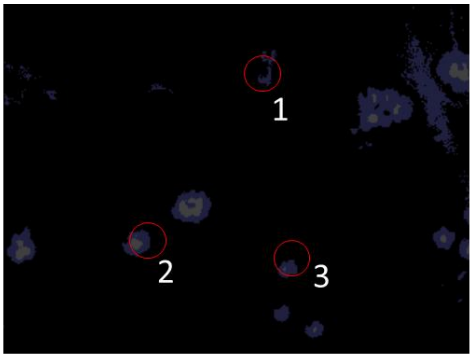
(-) ISL A549 cell region at 24 h



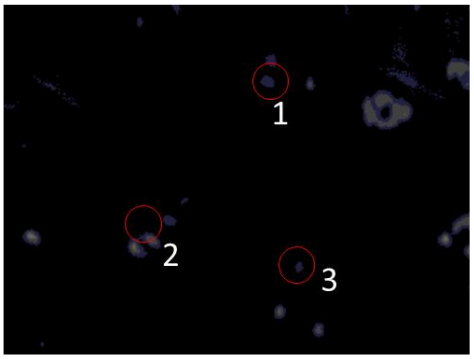
B



(+) 100 μM ISLA549 cell region 0 h



(+) 100 μM ISLA549 cell region 12 h



(+) 100 μM ISLA549 cell region 24 h

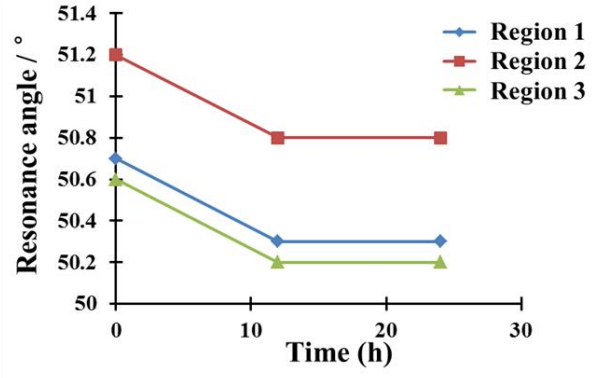


Fig. 4-6 A: The SPR images and time-course of resonance curves in the single cell ROI of A549 cell in the absence of ISL. **B:** The SPR images and time-course of resonance curves in the single cell ROI of A549 cell in the presence of 100 μM ISL.

Chapter 5

Conclusion and future work

5-1 Conclusion

In this doctoral course study, I have successfully achieved to evaluate a few effects of traditional natural medicines by using a high resolution of 2D-SPR instrument in real-time and simple manner. The anti-allergic effect of GA and ISL on individual RBL-2H3 cells was evaluated by the suppression of reflection intensity increase at cell region upon the antigen (DNP-BSA) stimulation. The experimental steps of 2D-SPR observation are less than β-hexosaminidase assay and did not use any reagents for degranulation assay. The 2D-SPR observation of cell response needed less than 1 hour. On the other hand, β-hexosaminidase assay needed more than 3 hours. Therefore, the mast cell-based 2D-SPR measurement is not only sensitive as same as β-hexosaminidase assay for screening natural medicines, but also it is easy, low cost and time saving. Besides, I could evaluate the anti-proliferation effect of ISL in A549 cells by 2D-SPR observation. The anti-proliferation effect of ISL on A549 cells was evaluated through resonance angle shift monitored by 2D-SPR observation. The resonance angle curves were almost no change in the presence of ISL within 48 hours through they shifted positively in the absence of ISL. Furthermore, proliferation and anti-proliferation process of cells was successfully monitored by SPR angle shift in single cell level. Thus cell-based 2D-SPR measurement might be also effective in evaluating the anti-cancer effect of natural products. This study suggested that

2D-SPR method might be promising to screen not only anti-allergic but also anti-cancer components of natural products, and also useful to discuss the intracellular signaling pathway and inhibition mechanism in individual cells.

5-2 Future work

In my Ph.D. study, I have successfully evaluated the anti-allergic and anti-proliferation effect of natural products (GA and ISL) by the 2D-SPR observation. It was demonstrated that 2D-SPR observation was a promising method not only to evaluate some effects of natural medicines but also to discuss the intracellular signaling pathway in mammalian cells. Therefore I would like to continue evaluating anti-cancer effect of natural products besides of ISL and discuss the intracellular mechanisms of these components with the high resolution 2D-SPR instrument.

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