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Development of novel monoclonal antibodies directed against catechins for investigation of antibacterial mechanism of catechins

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ABSTRACT

Catechins are major polyphenolic compounds of green tea. To investigate mechanism for antibacterial action of catechins, 11 monoclonal antibodies (MAbs) were raised against a 3-succinyl-epicatechin (EC)-keyhole limpet hemocyanin (KLH) conjugate. Amino acid sequences of variable regions determined for MAbs b-1058, b-1565, and b-2106 confirmed their innovative character. MAb b-1058 strongly interacted with its target substances in the following order of magnitude: theaflavin-3,3′-di-*O*-gallate (TFDG) > theaflavin-3-*O*-gallate (TF3G) ≥ theaflavin-3′-*O*-gallate (TF3'G) > gallocatechin gallate (GCg) > penta-*O*-galloyl-β-D-glucose (PGG) > epigallocatechin gallate (EGCg), as determined using surface plasmon resonance (SPR) on MAb-immobilized sensor chips. The affinity profiles of MAbs b-1058 and b-2106 to the various polyphenols tested suggested that flavan skeletons with both carbonyl oxygen and hydroxyl groups are important for this interaction to take place. *S. aureus* cells treated with EGCg showed green fluorescence around the cells after incubation with FITC-labeled MAb b-1058. The fluorescence intensity increased with increasing concentrations of EGCg. These MAbs are effective to investigate antibacterial mechanism of catechins and theaflavins.

1. Introduction

Catechins are major polyphenolic compounds of green tea. They show various physiological activities, such as antibacterial (Hamilton-Miller, 1995; Lee et al., 2009; Shiota et al., 1999), anticancer (Yang et al., 2002), and anti-inflammatory (Sano et al., 1999) properties. Catechins are more effective against Gram-positive than Gram-negative bacteria (Yoda et al., 2004), and epigallocatechin gallate (EGCg) and gallocatechin gallate (GCg), have been shown to have the highest antibacterial activity (Hamilton-Miller, 1995). Among the antipathogenic effects of catechins (Lee et al., 2009) is their capacity to reduce the minimum inhibitory concentration (MIC) of β-lactam antibiotics against methicillin-resistant *Staphylococcus aureus* (Shiota et al., 1999). A few mechanisms governing the antibacterial effect of catechins have been reported, such as damage to bacterial cells through binding to their surfaces (Ikigai et al., 1993) and the bactericidal action of hydrogen peroxide generated through catechin autooxidation (Arakawa et al., 2004; Akagawa et al., 2003). By using cerium chloride, we have shown that amounts of catechins adsorbed on the cell surfaces of Gram-positive bacteria, including *Staphylococcus aureus* and *Bacillus subtilis*, which are highly susceptible to catechins, are larger than those on Gram-negative bacteria, such as *Escherichia coli*, relatively resistant to catechins (Nakayama et al., 2011). Nakayama et al. (2013) reported that after treatment with EGCg, 16 proteins such as outer membrane porin protein C, penicillin binding protein 3, and Periplasmic L-asparaginase II, disappeared or showed markedly reduced intensity were identified in *E. coli* by two-dimensional electrophoresis. Of these, against porin protein, EGCg seems to enter into the porin pore and binds to Arg residues present on the inner surface of the pore channel through hydrogen bonding, resulting in inhibition of the porin function (Nakayama et al., 2013). On the other hand against *Bacillus subtilis*, 4 cell surface proteins, such as oligopeptide ABC transporter binding

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lipoprotein, glucose phosphotransferase system transporter protein, phosphate ABC transporter substrate-binding protein, and penicillin-binding protein 5 were identified by the same approaches (Nakayama et al., 2015). EGCg seems to bind to specific bacterial proteins to form strong aggregates that are resistant to solubilization by ionic detergents (Nakayama et al., 2012). However, the identities of most of the target proteins of EGCg in bacterial cells are still to be elucidated. To determine the localization of EGCg and the related polyphenols, specific direct detection and visualization methods are required. We reported a highly sensitive method to quantify EGCg using a specific EGCg-binding peptide, derived from a 67-kDa laminin receptor (Tachibana et al., 2004). The EGCg-binding peptide can also be used to visualize the localization of EGCg in and on bacterial cells. For this purpose, the peptide specific to GCg and EGCg must be labeled with a gold particle holding a fluorescent dye *via* a linker. However, this application requires a lot of work in terms of protocol development. A MALDI-imaging mass spectrometry method has previously been applied on different formalin-fixed paraffin-embedded human tissues (De Sio et al., 2015), showing that chemicals such as catechins could be detected, and their location visualized, using this imaging technique. However, this method cannot be used on bacterial cells as its maximum resolution ranges between 0.01 and 0.05 mm. Specific antibodies have been used to visualize the localization of target proteins and drugs in and on bacterial, plant, and animal cells. Since the methods employing specific antibodies as a device for the detection of antigens using electron and fluorescent microscopy are well established, specific antibodies are still regarded as important tools for sensitive and specific detection of targets of interest.

In this study, we describe the preparation and characterization of mouse monoclonal antibodies raised against 3-succinyl-epicatechin-keyhole limpet hemocyanin conjugate (3-succinyl-EC-KLH conjugate) and application to visualize binding of catechins to bacterial cells.

2. Materials and methods

2.1. Bacterial strains and polyphenols

Staphylococcus aureus NCTC 8325 was obtained from the National Collection of Type Cultures of Public Health England, Porton Down, Salisbury, SP4 0JG UK. *Escherichia coli* NBRC 3301 was obtained from the Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Kazusakamatari, Kisarazu-shi, Chiba, Japan. In order to test the reactivity of MAbs, catechin (C), gallocatechin (GC), catechin gallate (Cg), gallocatechin gallate (GCg), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECg), and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (PGG) were obtained from Sigma-Aldrich Japan K.K. Tokyo, Japan. Epigallocatechin gallate (EGCg), apigenin, curcumin, kaempferol, and nobiletin were obtained from Wako pure chemicals Co., Japan. Baicalein, butein, chalcone, gnetol, isoliquiritigenin, isorhapontigenin, myricitrin, neohesperidin dihydrochalcone, phlorizin, resveratrol, and rhapontigenin were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo Japan. Hesperetin, myricetin, quercetin, and tangeretin were obtained from Funakoshi Co., Ltd., Tokyo, Japan. Morin was a product from Kanto Chemical Co., Ltd., Tokyo Japan, rutin from ChlomaDex Co. Ltd., USA, luteolin from Tocris Bioscience, Co., Ltd., UK, and fisetin from LKT Laboratories, Inc., USA. Theaflavin (TF), theaflavin-3-*O*-gallate (TF3G), theaflavin-3′-*O*-gallate (TF3'G), and theaflavin-3, 3′-di-*O*-gallate (TFDG) were obtained from Nagara Science Co. Ltd., Gifu, Japan.

2.2. Preparation of antigen and monoclonal antibody

Epicatechin was succinylated and conjugated with keyhole limpet hemocyanin (KLH) according to the method described by Kawai et al. (2008). The 3-succinyl-EC-KLH conjugate was then immunized into BALB/c mice to prepare the monoclonal antibody (MAb).

2.3. Preparation of the immunogen and MAb

The preparation of MAb was consigned to BIO MATRIX RESEARCH, INC, Chiba, Japan. Six BALB/cAnNCrlCrlj mice were used for immunization. Spleen cells were fused with mouse myeloma cell line P3.X63.Ag.8.6.5.3 according to the method described by Köhler and Milstein (1975). Screening of the specific antibody producing hybridoma was done using 3-succinyl-EC-KLH conjugate as antigen immobilized onto each well of a microplate. MAbs were purified from the culture supernatant of the selected hybridomas using a protein G column. The immunoglobulin classes of the MAbs were determined using a MAb-typing kit (Sigma-Aldrich Japan K.K. Tokyo, Japan).

2.4. Labeling of MAbs with FITC

The selected MAbs were labeled with FITC using an NHS-Fluorescein antibody labeling kit (Thermo Fisher Scientific Inc. 3747 N Meridian Rd, Rockford, IL USA) according to the manufacturer's instruction.

2.5. Microplate ELISA assay for the estimation of MAbs reactivity

After investigating various reaction conditions, we established the following method for the estimation of antibodies' reactivity against polyphenols. First, 360 μL of 0.01 M Na Phosphate buffer (pH 7.0) containing 10 g/L bovine serum albumin (BSA) was added to each well of a 96-well microplate (high binding type, 400 ng/cm IgG, Nalge Nunc International), incubated overnight at 4 °C. Each well was then washed three times with 360 μL PBS containing 0.5 g/L Tween 20 (TPBS). To each well, 100 μL of 0.1 mg/L polyphenol dissolved in PBS (pH 7.4) was added and incubated for 2 h at 37 °C. The well was then washed three times with TPBS. 360 μL of 0.1 mg/L MAb dissolved in 0.01 M Na Phosphate buffer (pH 7.0) containing 10 g/L BSA was then added to the well and the plate was incubated for 1 h at 37 °C. As a blank, 0.01 M Na Phosphate buffer (pH 7.0) containing 10 g/L BSA was used instead of the MAb solution. The plate was washed three times with TPBS; after which, 360 μL anti-mouse IgG (Rabbit Anti-Mouse IgG-H & L (HRP), Abcam plc, Cambridge, UK) diluted to 1/4000 with PBS was added to the wells. After 1 h at 37 °C, the plate was then washed three times with TPBS after which 180 μL of chromogenic substrate solution was added and left to react at room temperature. The chromogenic substrate solution was prepared by mixing 0.2 M phosphate citrate buffer (0.2 M Na_2HPO_4 and 0.2 M $C_6H_8O_7{\cdot}H_2O$, pH 4.0) containing 0.3 g/L hydrogen peroxide with an equal volume of 0.6 mg/mL 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS, Sigma Aldrich). After 25 min, the reaction was stopped by adding 180 μL of 15 g/L oxalic acid, and absorbance was read at 405 nm using a microplate reader (Model 680, Bio-Rad). Results were shown as an average of absorbance obtained from triplicated wells.

2.6. Surface plasmon resonance assay

Biacore X (GE Healthcare, Uppsala, Sweden) was used for the surface plasmon resonance assays, according to the manufacturer's instruction. For the screening test of 6 MAbs against polyphenols, the MAbs were immobilized onto a sensor chip CM-5 (GE Healthcare, Uppsala, Sweden) through the amine coupling method, according to the protocol provided by the manufacturer. The concentrations of MAbs used for the immobilization ranged from 56 to 65 mg/L. Polyphenols at 100 mg/L in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20) were applied to the antibody-immobilized sensor chip at a 15 μL/min flow rate. The Sensorgram was monitored and the changes of the resonance units (RU) due to the binding of ligands to the immobilized MAbs were calculated. The polyphenols were dissociated from the antibodies applying 10 mM Gly-HCl buffer (pH 2.5). The same procedure was applied to achieve the