Original paper

Inhibitory Effects of Traditional Okinawan Vegetable Methanol Extracts and Their Primary Constituents on Histamine Release from Human Basophilic KU812 cells

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Received September 10, 2017; Accepted November 7, 2017

To clarify the effects of traditional Okinawan vegetable methanol extracts on histamine release from KU812 cells, we prepared methanol extracts from lyophilized powders of Crepidiastrum lanceolatum Nakai (hosoba-wadan), Peucedanum japonicum Thunb. (botan-bofu) and Artemisia indica Willd. var. orientalis (Pamp.) H. Hara (nishi-yomogi), and examined histamine concentrations released from cells by each extract. We also investigated the polyphenol profiles of each extract by HPLC and the impact on histamine release of the principle component. As a result, the histamine release from cells by hosoba-wadan and nishi-yomogi extracts were significantly suppressed (P < 0.05) when compared with control and botan-bofu extracts. Moreover, we found that the hosoba-wadan and nishi-yomogi extracts characteristically contained chlorogenic acid and luteolin-glycoside, respectively, and the histamine concentration released from the cells by the chlorogenic acid and luteolin were significantly lower (P < 0.05) than other polyphenols. Traditional Okinawan vegetable extracts suppressed histamine release from allergic cells, suggesting a new therapeutic approach for allergic diseases.

Keywords: histamine release, human basophilic KU812 cells, traditional Okinawan vegetable, chlorogenic acid, luteolin glycoside

Introduction

Okinawa is well known as a longevity prefecture in Japan. Therefore, it is of great interest to investigate the relationship between health or longevity and the consumption of distinctive Okinawan foods. In particular, there are many regionally specific agricultural crops in Okinawa. Among these, 28 agricultural crops are known as “traditional Okinawan agricultural crops (shima yasai)”, which are defined as follows: i) they have been used since before World War II, ii) they are commonly used in local cuisine, and iii) they are suitable for the climate of Okinawa. These crops contain relatively large amounts of polyphenols comprising several subtypes (Maeda 2009).

Moreover, in modern Okinawa, owing to the westernization of food culture, there have been decreases in consumption of traditional Okinawan crops, with corresponding reductions in life expectancy and increases life-style related and/or allergic diseases (Gavrilova et al., 2012; Sho 2001). Therefore, it is thought that there is a relationship between the consumption of traditional Okinawan crops and health or longevity.

Thus, many studies have shown that the physiological or biological functions, including anti-oxidant, anti-inflammatory, anti-allergy, anti-cancer and disease-ameliorating effects, of polyphenols derived from fruits and vegetables (Amarya et al., 2015; Pandey et al., 2009; Tangney et al., 2013). However, there...
have been few reports on the physiological effects of polyphenols found in traditional Okinawan vegetables.

In the present study, therefore, we focused on traditional Okinawan vegetables with distinct polyphenol profiles, such as Crepidastrum Lanceolatum Nakai (hosoba-wadan), Peucedanum Japonicum Thunb. (botan-bofu), and Artemisia indica Willd. var. orientalis (Pamp.) H. Hara (nishi-yomogi), and examined traditional Okinawan vegetable methanol extracts for their anti-allergic properties, using the histamine release assay with human basophilic KU812 cells. We found potent effects on the suppression of histamine release from KU812 cells by addition of hosoba-wadan and nishi-yomogi methanol extracts. Moreover, we analyzed in detail the components of hosoba-wadan and nishi-yomogi methanol extracts using HPLC, and assessed some of the key components present in both vegetable methanol extracts for their effects on histamine release from KU812 cells.

**Materials and Methods**

**Polyphenol preparation from traditional Okinawan vegetables**

Freeze-dried hosoba-wadan, nishi-yomogi and botan-bofu, cultivated at Okinawa Prefecture Agricultural Research Center in Japan, was extracted twice (50℃, 1 h) with methanol, and the solvent was evaporated. Finally, extracts were resolved in ethanol for the in vitro study.

**Determination of total polyphenol**

The total polyphenol content of vegetable methanol extracts was determined by the Folin-Denis method with minor modification (Folin et al., 1912). Briefly, a phenol reagent (20 μL), an aqueous solution of 10% Na₂CO₃ (40 μL) and distilled water (120 μL) were added to 20 μL of the methanol extract, and the mixture was incubated at room temperature for 1 h under dark conditions. The absorbance was measured at 750 nm with a spectrophotometer. An aqueous solution of gallic acid was used as a standard.

**Cell culture**

KU812 cells, obtained from the JCRB Cell Bank (JCRB0104, Osaka, Japan), were cultured in RPMI 1640 (Wako Pure Chemical Industries, Ltd. Osaka, Japan) containing 10% (v/v) fetal bovine serum (Sigma-Aldrich Co., St. Louis, MO) in a humidified atmosphere with 5% CO₂ at 37℃, and were subcultured for MTT assay and histamine release assay after 3 days. KU812 cells were cultured with culture medium containing the same volume of serum and Ca²⁺ (1.1 mM MgCl₂, 11.9 mM NaHCO₃, 2℃, 5 mM calcium ionophore A23187, and distilled water) and were suspended in 0.5 mL Tyrode buffer to give 2×10⁴ cells/mL. Next, 1 mM CaCl₂, 5 mM calcium ionophore A23187 (Sigma-Aldrich) and test sample solution (10% v/v) were added to the cell suspension. At the same time, we also prepared the blank group without addition of A23187. It was incubated at 37℃ for 20 min, and the reaction was stopped by cooling in an ice bath for 5 min. The cell suspension was centrifuged at 200×g for 5 min. The histamine concentration of the supernatant was determined according to the fluorometric method (Shore 1959).

The inhibition of histamine release from KU812 cells was calculated using the following equation:

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\text{Percentage of histamine release inhibition (\%)} = 100 \times \frac{(1 - (S - N))}{P - N}
\]

Where S is the histamine content released from cells incubated with the test sample and with A23187, N is the histamine content released from cells spontaneously without A23187 or test sample, and P is the histamine content released from cells incubated with A23187 without test sample. All measurements were obtained by subtracting the blank value.

**HPLC analysis of polyphenolic composition of hosoba-wadan and nishi-yomogi methanol extracts**

Polyphenols in the hosoba-wadan and nishi-yomogi methanol extracts were determined by HPLC, using a Mightysil RP-18 GP II (5 μm) column (4.6 mm × 250 mm; Kanto Chemical CO., INC.) at a flow rate of 1.0 mL/min. The sample was injected, and elution was performed with a system composed of solvent A (0.1% phosphoric acid in water) and solvent B (0.1% TFA in acetonitrile) mixed at the same ratio (50:50). The elution was detected by UV-VIS detector at 330 nm. Compounds corresponding to each peak were identified by comparison with the retention time of reference standards and our previous report (Maeda et al., 2006). Moreover, we estimated the amounts of polyphenolic constituents using the external standard method with chlorogenic acid (Wako Pure Chemical Industry, Ltd. Osaka, Japan).

**Statistical analysis**

Data are expressed as means ± SEM (standard errors of the mean). In the case of MTT assay, data were assessed by one-way analysis of variance (ANOVA), and then each value was compared with that of control group by Dunnett test. For other results, Tukey-Kramer test was used for comparisons between groups. Differences were considered to be significant at \( P < 0.05 \).
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Results

Polyphenol contents of traditional Okinawan vegetables

Polyphenol contents of traditional Okinawan vegetable methanol extracts were measured by the Folin-Denis method. The polyphenol contents of hosoba-wadan, nishi-yomogi and botan-bofu were 17.9, 96.1 and 41.3 mg/g leaf, respectively. Among the 3 vegetables, nishi-yomogi contained the largest amounts of polyphenol. This trend was also observed in our previous studies. Therefore, these vegetable methanol extracts were redissolved in ethanol and used at the same concentration in the subsequent series of experiments.

Effects of traditional Okinawan vegetable methanol extracts on cell viability

KU812 cell viability after addition of hosoba-wadan, nishi-yomogi and botan-bofu methanol extracts is shown in Fig. 1. Cell viability or cytotoxicity was assessed by MTT assay, in which a tetrazolium salt is reduced to formazan in living cells. Cell viability was reduced in a concentration-dependent manner with all vegetable extracts. The methanol extract of hosoba-wadan was not cytotoxic at concentrations below 5 μg/mL, while nishi-yomogi and botan-bofu significantly reduced cell viability at 5 μg/mL as compared with controls. Moreover, the addition of 1 μg/mL extracts did not show any marked cytotoxicity, including cell desquamation under microscopic observation (data not shown). Based on these results, all extracts were prepared at a concentration of 1 μg/mL ethanol and were added to assess their influence on histamine release from KU812 cells.

Effects of traditional Okinawan vegetable methanol extracts on histamine release from KU812 cells

The effects of methanol extracts on histamine release from KU812 cells are shown in Fig. 2. On addition of 1 μg/mL hosoba-wadan and nishi-yomogi methanol extracts, the amounts of histamine released from KU812 cells were significantly lower ($P < 0.05$) as compared to those with botan-bofu and controls. In addition, the histamine release inhibition rate of hosoba-wadan and nishi-yomogi were 42.5 and 42.8%, respectively. In particular, the histamine release inhibitory effects of hosoba-wadan methanol extract were reproducibly observed (data not shown).

Polyphenol profiles of Hosoba-wadan and nishi-yomogi methanol extracts by HPLC analysis

The polyphenol profiles of hosoba-wadan and nishi-yomogi methanol extracts were analyzed by HPLC (Fig. 3). The primary components of hosoba-wadan methanol extract were chlorogenic acid, chicoric acid and luteolin glycosides, such as luteolin 7-O-β-D-glucopyranoside and luteolin 7-O-β-D-glucuronide. While, those of nishi-yomogi methanol extract were chlorogenic acid, caffeoylquinic acid, and isochlorogenic acids, such as 3,5-, 4,5- and 3,4-dicaffeoylquinic acid. In addition, Table 1 shows the quantitative values of polyphenols present in methanol extracts of hosoba-wadan and nishi-yomogi.

Effects of principal polyphenols present in hosoba-wadan and nishi-yomogi on histamine release from KU812 cells

In order to examine the effects of the principal polyphenols on histamine release from KU812, we carried out histamine release assay with the addition of chlorogenic acid, chicoric acid, luteolin, caffeic acid and quinic acid. We used luteolin, caffeic acid and quinic acid, because the luteolin present in hosoba-wadan as a glycoside is hydrolyzed in vivo to be an aglycone, and chlorogenic acid is...
Histamine concentration released from human basophil KU812 cells by addition of 1 µg/mL methanol extracts of traditional Okinawan vegetables. Data are shown as means ± SEM of four treatments. Comparison between groups was conducted by Tukey-Kramer test, and different letters were to be considered statistically significant differences between groups at $P < 0.05$.

Fig. 2. Polyphenol profile of hosoba-wadan (A) and nishi-yomogi (B) methanol extracts by HPLC analysis. Extracts were analyzed by high performance liquid chromatography (HPLC) equipped with MightySil RP-18 GP II column and UV-VIS detector. Each peak was identified with the retention time of reference standards and our previous report (Maeda et al., 2006).
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we observed significantly lower (P < 0.05) histamine release from KU812 cells after addition of chlorogenic acid and luteolin when compared with control, caffeic, quinic and chicolic acid (Fig. 4). In addition, the inhibition rates of histamine release by chlorogenic acid and luteolin were 64.5 and 76.7%, respectively (Fig. 5). In this study, the suppressive effects of histamine release by luteolin were the greatest among the groups.

Discussion

In the present study, we first examined the influence of methanol extracts of three traditional Okinawan vegetables (hosoba-wadan, nishi-yomogi and botan-bofu) on histamine release from human basophilic KU812 cells. As a result, we found effective suppression of histamine release from KU812 cells by hosoba-wadan and nishi-yomogi methanol extracts. The polyphenol profiles of hosoba-wadan and nishi-yomogi using HPLC revealed that these vegetables mainly contain chlorogenic acid, chicoric acid and luteolin glycosides, respectively. The inhibition of histamine release by addition of primary constituent polyphenols present in hosoba-wadan and nishi-yomogi or their possible decomposition products was then estimated. Among the samples added to the cells, chlorogenic acid and luteolin exhibited the most prominent inhibition of histamine release from the cells. In particular, we

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### Table 1. Quantitative value of polyphenol constituting methanol extracts of hosoba-wadan and nishi-yomogi.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Chlorogenic acid</th>
<th>Chicoric acid</th>
<th>Luteolin 7-O-β-D-glucopyranoside</th>
<th>Luteolin 7-O-β-D-glucuronide</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. lanceolatum Nakai</td>
<td>0.90</td>
<td>7.85</td>
<td>1.03</td>
<td>2.63</td>
</tr>
<tr>
<td>A. indica Wild. var. orientalis (Pamp.) H. Hara</td>
<td>28.0</td>
<td>7.93</td>
<td>23.3</td>
<td>30.5</td>
</tr>
</tbody>
</table>

Each value is expressed as chlorogenic acid equivalent.

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![Fig. 4.](image-url) Histamine concentration released from human basophil KU812 cells by addition of aglycones constituting the polyphenols present in each vegetable methanol extracts. Each polyphenol was prepared at the concentration of 3.5 µM. Data are shown as means ± SEM for three treatments. Comparison between groups was conducted by Tukey-Kramer test, and different letters were to be considered statistically significant differences between groups at P < 0.05.
found that luteolin showed the strongest suppressive effects on histamine release from the cells. Based on these data, we expect that the daily consumption of foodstuffs containing functional polyphenols may contribute to maintaining human health.

Type I allergies, including anaphylaxis, are caused by histamine released from mast cells. That is, binding of IgE antibodies and antigens to FcεRI receptors on mast cell membranes causes an elevation of intracellular calcium ion (Ca^{2+}) concentration and releases histamine from cells (Amin 2012; Dombrovicz et al., 1993; Kinet et al., 1991; Kinet 1999; Gauchat et al., 1993; Metzger 1991). Therefore, these events are very important for histamine release. It is also known that mast cells and human basophilic KU812 cells are associated with allergic reactions through chemical mediators such as histamine (Yamada et al., 2010). In fact, histamine release from these cells is an important evaluation issue for estimating the suppressive effects of functional ingredients against allergies. In the present study, hosoba-wadan and nishi-yomogi methanol extracts effectively inhibited histamine release from KU812 cells as compared with those of other groups (Fig. 2). These results indicate the possibility of anti-allergic action due to the suppressive effects of the hosoba-wadan and nishi-yomogi polyphenols on histamine release from cells. Moreover, in addition to the in vitro study using KU812 cells, we also observed similar suppressive effects on histamine release from RBL 2H3 cells by the hosoba-wadan and nishi-yomogi methanol extracts (data not shown).

Next, in order to examine the function of the polyphenols present in hosoba-wadan and nishi-yomogi, we performed HPLC analysis to clarify the major polyphenols and investigated the influence of each polyphenol on histamine release from KU812 cells. As shown in Fig. 3, we found that the primary polyphenols of hosoba-wadan were luteolin glycosides, and those of nishi-yomogi were chlorogenic acid, chicoric acid and isochlorogenic acids. In general, it is thought that the glycoside is hydrolyzed and absorbed into saccharides and aglycones in vivo (D’Archivio et al., 2007; Yasuda et al., 2010; Day et al., 2001). For that reason, we used luteolin, chicoric acid, chlorogenic acid, and its constituents, caffeic acid and quinic acid, to clarify the suppressive effects on histamine release of each polyphenol or component. Among the polyphenols and components used in this study, both luteolin and chlorogenic acid inhibited the histamine release from cells (Fig. 5).

Chlorogenic acid is a type of polyphenol widely distributed in coffee, fruits and vegetables. There are many investigations on the biological functions of chlorogenic acid, in which chlorogenic acid showed anti-oxidative, anti-inflammatory, anti-cancer, anti-viral and anti-allergic effects (Pandey et al., 2009). Moreover, it has been also reported that the natural products present in chlorogenic acid inhibit histamine release from mast cells (Qin et al., 2010). It has also been reported that chlorogenic acid induces degranulation of peritoneal mast cells (Huang et al., 2010). Although there have been conflicting reports on chlorogenic acid as described above, the physiological function of chlorogenic acid might depend on its presence in vegetables or fruits. From the present data, chlorogenic acid is actually expected to have anti-allergic effects although there may be differences in degree. On the other hand, luteolin is known to be an anti-allergic flavonoid. Kimata et al. reported that luteolin inhibited the release of chemical mediators such as histamine and cytokines involved in allergies from the activated mast cells through inhibition of the IgE-mediated reaction (Kimata et al., 2000).

Interestingly, the mechanisms of the anti-allergic function of polyphenols is thought to depend on their molecular weight. Kondo et al. and Tomochika et al. reported that the high-molecular-weight polyphenols, such as procyanidines and high-molecular-weight peanut-skin extract, can inhibit the degranulation of cells by binding to the mast cell surface (Kondo et al., 2006; Tomochika et al., 2011). In their reports, they noted that the high-molecular-weight polyphenols cover the surface of mast cells and inhibit binding of antibody and antigen, thereby suppressing release of histamine, which is the cause of allergies. On the other hand, low-molecular-weight polyphenols are thought to inhibit the release of histamine by a mechanism of action that differs from that of high-molecular-weight polyphenols. In the present study, as shown in Fig. 4, we made a similar observation. That is, the low-molecular-weight polyphenols, such as caffeic and quinic acid constituting...
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chlorogenic acid, could not inhibit release of histamine from cells, while chlorogenic acid was able to inhibit the release of histamine from KU812 cells. The effects of chlorogenic acid may be due to a mechanism of action based on molecular weight differences.

In summary, we investigated the influence of methanol extracts of traditional Okinawan vegetables, which are rich in polyphenols, on histamine release from human basophil KU812 cells. We observed the effective suppression of histamine release from cells by addition of hosoba-wadan and nishi-yomogi methanol extracts. Moreover, chlorogenic acid and luteolin among the polyphenols present in the extracts exhibited the most prominent inhibition of histamine release from the cells. The habitual usage of traditional Okinawan vegetables, such as hosoba-wadan and nishi-yomogi, could be expected to contribute to the alleviation of allergies. Irrespective, regulation of the production of histamine from cells related to allergies by consumption of traditional Okinawan vegetables with functional polyphenols might be a beneficial target for allergy relief. Therefore, we believe that further study will be necessary to clarify the physiological benefits using animal models.

References