Note

Hot-Water Extracts from Adzuki Beans (Vigna angularis) Suppress Not Only the Proliferation of KATO III Cells in Culture but Also Benzo(a)pyrene-Induced Tumorigenesis in Mouse Fore stomach

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Summary Treatment of human stomach cancer KATO III cells with hot-water extracts from adzuki beans led to their growth inhibition as well as apoptosis induction. There are morphological changes in the cultured cells treated with the extracts, by which DNA fragmentation characteristic of apoptosis was actualized both concentration- and time-de pendently. In contrast, N-acetyl-L-cysteine suppressed such DNA fragmentation, implying that the extracts from adzuki beans might exert antitumorigenicity via active oxygen-induced apoptosis. In order to verify this hypothesis in animal experiment, the 40% ethanol fraction of hot-water extracts was examined for its preventive effect against benzo(a)pyrene-induced tumorigenesis in the forestomach of A/J mice, given as drinking water containing the above fraction at 0.5–2.0% levels. Consequently, forestomach cancer has turned out to be reduced by 36–62% in tumor weight relative to the control. These results suggest that the fraction of hot-water adzuki extracts may serve as a nutrapharmaceutical or functional food available for cancer prevention.

Key Words adzuki beans (Vigna angularis), cultured KATO III cells, benzo(a)pyrene-induced forestomach cancer, antitumorigenicity

Adzuki beans (Vigna angularis) are a very important food material in East Asia. In traditional Chinese medicine, adzuki beans familiar to “chi xiao dou” (Phaseoli semen) have been used habitually for multi-purposes including diuretics and antidotes, as well as for symptoms of dropsy and beriberi (1). In Japan, adzuki beans are used mainly for the production of confectioneries such as youkan, manjuu, amanatoo, etc. (2–8). Other uses include cosmetics (facial cream or shampoo), medical supplies, nitrogen-fixing green manure, livestock feed and soil stabilizer (2–5, 9–13). In the case of food processing, heat treatment of adzuki beans results in a large amount of hot-water extracts (dipping water), which is usually discarded. This study was conducted to find out merits in the function of these hot-water extracts from a cancer-preventive point of view.

Materials and Methods

Fractionation of hot-water extracts from adzuki beans. A common species of adzuki beans were harvested in Tokachi, Hokkaido, and were used for the production of a bean jam, a traditional Japanese food. The adzuki beans were boiled in a cooker prior to preparation of the bean jam. Hot-water extracts (dipping water) were obtained in a large amount. Hence, hot-water extracts were concentrated, applied to a DIAION HP-20 (column size φ5 mm×300 mm) and eluted stepwise with distilled water, 40%, 60%, and 80% ethanol. The respective fractions were evaporated to dryness and hereinafter referred to as Fract. 1, 2, 3 or 4 in their order of elution.

Culture of human stomach cancer KATO III cells. KATO III cells were provided by the American Type Culture Collection (ATCC) and the Health Science Research Resources Bank (HSRRB), Osaka, Japan. The KATO III cells were grown at 37°C in a combination medium of RPMI 1640 medium (45%), Eagle’s MEM (45%), and fetal calf serum (10%), penicillin G (50 IU/mL) and streptomycin (50 μg/mL) under 95% humidity and a 5% CO2 atmosphere. Medium renewal was carried out every 7 d, and no contamination with mycoplasma was checked on any occasion.

Preparation of human lymphocyte cells. Three milliliters of lymphocyte separation medium was transferred to a centrifuge tube, on which a 1:1 mixture of heparinized blood and physiological saline was layered under aseptic conditions. The tube was centrifuged at 400×g at room temperature for 20 min. After removal of clear plasma, the lymphocytes and its equal volume of PBS (−) were transferred to a new centrifuge tube, followed by centrifugation for 10 min at 2600×g at room temperature. The resulting lymphocytes were washed with...
PBS (-), and then suspended in RPMI 1640 containing 10% fetal calf serum and 2% phytohemagglutinin-M.

**Morphological change in KATO III cells.** Exponentially growing KATO III cells were inoculated into a culture medium at a density of $3 \times 10^5$ cells/mL and cultured for 2 d in the absence or presence of each Fract. sample at 2 mg/mL. The respective cells were examined for their morphological changes using an epifluorescence microscope with a cooled CCD camera digital imaging system and Fuji picrography 3000 as described by Okumura et al. (14).

**Analysis of DNA fragmentation by electrophoresis.** Exponentially growing KATO III cells were inoculated at density of $3-4 \times 10^5$ cells/mL. After culturing for 1-3 d in the presence of each Fract. sample, the cells were harvested by centrifugation at 800×g, from which DNA was isolated as described previously (15). The resulting DNA (in 2 µg) were applied to a 2% agarose gel and electrophoresed in 40 mM Tris-acetate buffer of pH 7.5 containing 2 mM EDTA.

**Flow cytometric analysis of cell death.** The DNA content of Fract. 2-treated KATO III cells was determined using a Becton Dickinson Immunocytometer and a Cycle TEST™ plus DNA reagent kit. Briefly, the cell pellets (5×10^6 cells) were suspended in 0.25 mL of solution A (trypsin buffer) and incubated at 25°C for 10 min, to which 200 µL of solution B (trypsin inhibitor) was added and incubated at 25°C for 10 min. Then 200 µL of solution C (propidium iodide) was added and incubated at 4°C for 10 min. After filtration of the supernatant through a 50 µm nylon mesh, the DNA content of the stained nuclei was flow-cytometrically analyzed with a Becton Dickinson apparatus. The distribution of DNA content was obtained as percentage of hypodiploid cells (pre G1) in the cells distribution with DNA content less than G1.

**Effects of caspase inhibitors and N-acetyl-L-cysteine on Fract 2-induced DNA fragmentation of KATO III cells.** Exponentially growing KATO III cells were inoculated at a density of $3-4 \times 10^5$ cells/mL. Fract. 2 was added to the KATO III cells which had been pre-incubated for 2 h in the presence of caspase inhibitors, such as Z-VAD-FMK, Ac-DEVD-CHO and Z-Asp-CH₂, followed by culturing for 3 d. Since then, DNA was isolated from the cell pellets of harvested cells and in 2 µg portions applied to a 2% agarose gel for electrophoresis. On the other hand, KATO III cells were inoculated at a density of $3-4 \times 10^5$ cells/mL. After 2 h pre-incubation at a 5 mM concentration of N-acetyl-L-cysteine, the cells were cultured with Fract. 2 (2 mg/mL) for a further 3 d under the usual condition, from which DNA was isolated and subjected to electrophoresis as above.

**Animal experiment on cancer prevention of Fract. 2.** Benzo(a)pyrene-induced tumorigenesis in the forestomach of A/J mice was performed according to a slight modification of the procedure described by Wattenberg (16). In brief, female A/J mice of 5-6 wk age were divided into five groups and given free access to drinking water containing 0, 0.5, 1.0 and 2.0% (w/v) of Fract. 2 or 0.8% (w/v) of hot-water extracts, to which benzo(a)pyrene (in 1.5 mg/100 µL of corn oil) was administered by gastric intubation once a week for periods of initial 4 wk. All the mice were housed in an air-conditioned room with a 12 h light/dark cycle and fed MF pellets (Japan SLC, Ltd., Hamamatsu, Japan) through the experimental period. The mice were sacrificed 23 wk after the last administration of benzo(a)pyrene, so as to excise the stomach and weigh tumors generated there.

**Statistical analysis** All data were first analyzed by one-way ANOVA, and subsequently by Duncan’s multiple-range test, so that the differences among means were considered significant at $p<0.05$.

**Results and Discussion**

Figure 1 shows the inhibitory effects of Fract. 1-4 samples on the proliferation of KATO III cells. Above all, Fract. 2 exerted a remarkable inhibition. In this connection, it was investigated whether part of the reason might possibly be accounted by apoptosis. Figures 2 and 3 compare morphological changes in KATO III cells cultured for 3 d in the absence and presence of Fract. 2. Apoptotic bodies did not appear in the former but appeared in the latter (Fig. 2). Simultaneously, a DNA ladder was confirmed to occur in this case (Fig. 3A). DNA fragmentation in KATO III cells treated with Fract. 2 increased with the elapse of time (Fig. 3B) and concentration-dependently (Fig. 3C). It is necessary to quantify the extent of apoptosis to evaluate the obstruction of cancer cells by Fract. 2. Such an attempt was achieved by flow cytometry (Fig. 4). In practice, treatment of KATO III cells for 3 d with Fract. 2 at 1, 2 and 4 mg/mL brought about 25.7, 47.2 and 71.4% (hypodiploid phase), respectively. The obstruction of prolifera-

![Fig. 1. Proliferation of human stomach cancer KATO III cells and its inhibition by fractions of hot-water extracts from adzuki beans. Culture conditions of KATO III cells and fractionation of hot-water extracts were described in Materials and Methods. Cell culturing was carried out in the absence or presence of 2 mg/mL, Fract. 1, 2, 3 or 4. ◆: Control, □: Fract. 1, ○: Fract. 2, △: Fract. 3, ▲: Fract. 4.](image-url)
Antitumorigenicity of Hot-Water Extracts from Adzuki Beans

Fig. 2. Morphological changes in KATO III cells treated with Fract. 2. The cells were cultured in the absence (A) or presence of Fract. 2 (B) for 3 d under the same conditions as above, and observed for morphological change using an epifluorescence microscope equipped with cooled CCD camera (Phometrics, PXL 1400) digital imaging system and Fuji pictography 3000.

Fig. 3. DNA fragmentation in KATO III cells treated with Fract. 1, 2, 3, or 4. (A) The cells were cultured for 3 d in the absence (lane 1) or presence of 2 mg/mL Fract. 1 (lane 2), Fract. 2 (lane 3), Fract. 3 (lane 4) and Fract. 4 (lane 5). (B) The cells were cultured in the absence of fractions for 3 d (lane 1) or in the presence of 2 mg/mL Fract. 2 for 1 d (lane 2), 2 d (lane 3), and 3 d (lane 4). (C) The cells were cultured for 3 d in the absence of fractions (lane 1) or in the presence of Fract. 2 at 1 mg/mL (lane 2), 2 mg/mL (lane 3), and 3 mg/mL (lane 4). DNA isolated from these cells (in 2 µg) was applied to a 2% agarose gel and electrophoresed in 40 mM Tris-acetic acetate buffer at pH 7.5 containing 2 mM EDTA as above-mentioned. M represents λDNA digested with HindIII.

Addition of KATO III cells by this fraction may be interpreted in relation to cell cycle. Incidentally the percentage of G0/G1 cells was 36.3% in non-treated KATO III cells, but 22.1% in those treated with 1 mg/mL of Fract. 2, suggesting that the inhibition of cell growth might be associated with G0/G1 stage. In contrast, the addition of 2 mg/mL of Fract. 2 to normal leukocytes from healthy volunteers didn’t induce apoptosis (data not shown). Active substance(s) is/are dissolved in water and adsorbed on HP-20 resins, easy to bind selectively hydrophobic compounds. Hot-water extracts from adzuki beans include saponin, tannins, catechin and so on. Catechin-dimer or -trimmer, of black bean seed coat origin, induces apoptosis in human promyelotic leukemia Molt4B cells (17). The color of hot-water extracts from adzuki beans turned dark-brown, probably because of catechin, which might induce apoptosis.

In one case, apoptosis proceeds via a cascade being
Fig. 4. DNA contents in KATO III cells without and with treatment of Fract. 2. The cells which had been cultured for 3 d without (A) and with Fract. 2 at 1 mg/mL (B), 2 mg/mL (C) or 4 mg/mL (D), were harvested, fixed and stained with propidium iodide (PI). The DNA content was analyzed by flow cytometry with a Becton-Dickinson FACS. The number of hypodiploid cells (Pre-G1 phase) is expressed as percentage of total cells.

Table 1. Preventive effects of Fract. 2 intake against benzo(a)pyrene-induced tumorigenesis in the mouse forestomach.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>No. of tumors</th>
<th>Tumor weight (g)</th>
<th>Arrest (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle (control)</td>
<td>5.80±1.10a</td>
<td>0.21±0.06a</td>
<td>16.8</td>
</tr>
<tr>
<td>2</td>
<td>Whole extracts, 8 mg/mL</td>
<td>5.38±2.00ab</td>
<td>0.18±0.07ab</td>
<td>60.3</td>
</tr>
<tr>
<td>3</td>
<td>Fraction 2, 5 mg/mL</td>
<td>5.50±2.43ab</td>
<td>0.14±0.05bc</td>
<td>62.1</td>
</tr>
<tr>
<td>4</td>
<td>Fraction 2, 10 mg/mL</td>
<td>3.33±0.52b</td>
<td>0.09±0.02c</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fraction 2, 20 mg/mL</td>
<td>3.75±1.28ab</td>
<td>0.08±0.02c</td>
<td></td>
</tr>
</tbody>
</table>

One-hundred microliters of oily solution containing 1.5 mg benzo(a)pyrene was administered by gastric intubation once a week in a 4-wk period to A/J mice given either whole extracts (8 mg/mL) or Fract. 2 (5, 10, 20 mg/mL) as drinking water. At 23 wk after the last ingestion of benzo(a)pyrene, each stomach was excised from all the mice to measure the number and weight of tumors. Value are the means±SD (n=10). Those not sharing a common superscript letter are significantly different at p<0.05 by Duncan's multiple range test.

pathway in which the caspase activated upstream activates the down-stream caspases directly or indirectly, attended with DNA fragmentation. A question to arise is whether caspase inhibitors such as Z-VAD-FMK, Ac-DEVD-CHO and Z-Asp-CH2 affect DNA fragmentation by Fract. 2. The experimental result revealed that any caspase inhibitors in 100 μM had no effect in disturbing the apoptosis of KATO III cells by 2 mg/mL Fract. 2 (data not shown). As described by Susin et al. (18), a fall in mitochondrial membrane potential accompanies the formation of by-products including apoptosis-inductive factor(s). Among them is active oxygen serving as a potent factor capable of altering the character of mitochondrial membrane, which is attacked by N-acetyl-t-cysteine. Fract. 2-induced DNA fragmentation of KATO III cells was actually improved to some extent in the coexistence of N-acetyl-t-cysteine (data not shown).

Table 1 summarizes the preventive effects of Fract. 2 administered to A/J mice against benzo(a)pyrene-induced tumorigenesis in the forestomach. The number and weight of tumors were evaluated at the 23 wk after the final administration of benzo(a)pyrene. Both values measured were significantly lower in the group given 1.0% Fract. 2 as drinking water relative to the control. A similar effect was observed for the group given 2.0% Fract. 2 as well. This finding supports the possibility that antitumorogenic component(s) predominate(s) in Fract. 2. Therefore, we need to clarify the substance in...
itself for what exerts cancer prevention. In addition to that, it remains to be further elucidated in what manner active oxygen generates from Fract. 2 and triggers the initiation of apoptosis in combination with other factors.

REFERENCES


