Inhibitory Effects of Naringenin on Tumor Growth in Human Cancer Cell Lines and Sarcoma S-180-Implanted Mice

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We have investigated the effect of naringenin (NGEN) on tumor growth in various human cancer cell lines and sarcoma S-180-implanted mice. NGEN showed cytotoxicity in cell lines derived from cancer of the breast (MCF-7, MDA-MB-231), stomach (KATOIII, MKN-7), liver (HepG2, Hep3B, Huh7), cervix (Hela, Hela-TG), pancreas (PK-1), and colon (Caco-2) as well as leukemia (HL-60, NALM-6, Jurkat, U937). NGEN-induced cytotoxicity was low in Caco-2 and high in leukemia cells compared to other cell lines. NGEN dose-dependently induced apoptosis, with hypodiploid cells detected in both Caco-2 and HL-60 by flow cytometric analysis. In vitro, NGEN inhibited tumor growth in sarcoma S-180-implanted mice, following intraperitoneal or peroral injection once a day for 5 d. Naringin (NG) also inhibited tumor growth by peroral injection but not intraperitoneal injection. NGEN, one of the most abundant flavonoids in citrus fruits, may have a potentially useful inhibitory effect on tumor growth.

Key words naringenin; naringin; apoptosis; human cancer cell line; sarcoma S-180

MATERIALS AND METHODS

Cell Lines and Cell Culture Human breast cancer MDA-MB-231 cells and colon cancer Caco-2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.) and Riken Cell Bank (Tsukuba, Japan), respectively. The other cell lines were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). All cells were routinely kept in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin G (100 U/ml)/streptomycin (100 μg/ml) at 37°C in a humidified 5% CO2–95% air incubator under standard conditions.9) Cell viability was measured by counting, excluding those cells stained with 0.2% trypan blue. To maintain exponential growth, cells were seeded at 1×10^5 cells/ml and passaged every 4 to 5 d.

Chemicals and Animals Naringin (NG), Naringenin (NGEN) and the other reagents were supplied by either Sigma (St. Louis, MO, U.S.A.) or Nacalai Tesque (Kyoto, Japan) and of the highest grade available. All cell culture reagents were obtained from Invitrogen Corp (Carlsbad, CA, U.S.A.). For the experiments in vivo, NG and NGEN were dissolved in 0.5% Tween 80 in saline. For the experiments in vitro, NGEN was dissolved in dimethylsulfoxide (DMSO) to make a concentration of 400 mM as a stock solution. It was used after dilution of the stock solution with DMSO. DMSO at concentrations lower than 0.5% had no effect on cell growth. Exposure to light was kept to a minimum for all drugs used. Male ddY mice (6 weeks old, 30–32 g) obtained from Japan SLC (Hamamatsu, Japan) were maintained on a 12 h light/dark cycle in a temperature- and humidity-controlled room. The experiments were conducted in accordance with the standards established by the Japanese Pharmacological Society. The animals were allowed free access to laboratory pellet chow (CE-2; CLEA Japan Inc., Tokyo, Japan) and water before the experiments.

Cytotoxicity Cytotoxicity was assessed by MTT staining as described by Mosmann.10) The cells were plated (2×10^4

Fig. 1. Structure of Naringin (NG) and Naringenin (NGEN).
cells/well) in 96-well plates for 24 h and treated with different concentrations of NGEN for 48 h. After the treatment, 10 μl of the tetrazolium compound MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added for 2 h at 37 °C. The cells were then and centrifuged (3000×g for 5 min), and the supernatant was aspirated. The cells were lysed and solubilized by the addition of 100 μl of 0.4 N HCl in isopropanol. The absorbance of each well was determined at 590 nm using an Intermed model NJ-2300 Microplate Reader. Survival (%) was calculated relative to the control.

Flow Cytometric Analysis The cells were cultured in triplicate in 35 mm dishes at a concentration of 4×10^4 cells. After 24 h, cells were treated with 100, 250, 500 or 1000 μM naringenin (NGEN), in parallel with cells grown in the absence of NGEN to determine effects on apoptotic characteristic, hypodiploid cells (sub-G1 peak). After the culture, the cells were washed with phosphate-buffered saline (PBS) and collected. Cell suspensions were filtered through a 60 μm mesh filter. Data acquisition and analysis were performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

In Vivo Antitumor Effect of Naringenin Sarcoma S-180 cells (1×10^6) in 0.1 ml of PBS were subcutaneously injected between the femur of the male ddY mice. Tumor-bearing mice were subdivided into groups of 10. After 24 h, mice were treated with naringin (NG) or naringenin (NGEN) as an intraperitoneal (i.p.) or peroral (p.o.) injection at a dose of 30, 100 or 300 mg/kg once a day for 5 d (5 times). Similarly, 5-fluorouracil (5-FU, 10 mg/kg, i.p.) was administered as a reference treatment. The comparative cytotoxicity is expressed as the 50% effective concentration (EC_{50}).

Table 1 Naringenin-Induced Cytotoxicity in Human Cancer Cell Lines

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cell name</th>
<th>Characteristics</th>
<th>48 h (μM; EC_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>MCF-7</td>
<td>ER (+), PgR (+)</td>
<td>240±23</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231</td>
<td>ER (-), PgR (-)</td>
<td>267±59</td>
</tr>
<tr>
<td>Stomach</td>
<td>KATO III</td>
<td>p53 (-)</td>
<td>407±58</td>
</tr>
<tr>
<td></td>
<td>MKN-7</td>
<td>p53 (mt)</td>
<td>320±40</td>
</tr>
<tr>
<td>Liver</td>
<td>HepG2</td>
<td>p53 (wt)</td>
<td>340±92</td>
</tr>
<tr>
<td></td>
<td>Hep3B</td>
<td>p53 (-)</td>
<td>230±51</td>
</tr>
<tr>
<td></td>
<td>Huh7</td>
<td>p53 (mt)</td>
<td>350±68</td>
</tr>
<tr>
<td>Cervix</td>
<td>Hela</td>
<td></td>
<td>243±22</td>
</tr>
<tr>
<td></td>
<td>Hela-TG</td>
<td>6-Thioguanine resistant</td>
<td>223±47</td>
</tr>
<tr>
<td>Pancreas</td>
<td>PK-1</td>
<td></td>
<td>237±43</td>
</tr>
<tr>
<td>Colon</td>
<td>Caco-2</td>
<td></td>
<td>557±48</td>
</tr>
<tr>
<td>Leukocyte</td>
<td>HL-60</td>
<td>Promyelocytic</td>
<td>153±15</td>
</tr>
<tr>
<td></td>
<td>NALM-6</td>
<td>B cell</td>
<td>157±16</td>
</tr>
<tr>
<td></td>
<td>Jurkat</td>
<td>T cell</td>
<td>206±50</td>
</tr>
<tr>
<td></td>
<td>U937</td>
<td>Diffuse histiocytic lymphoma</td>
<td>190±50</td>
</tr>
</tbody>
</table>

Cytotoxicity was estimated by MTT assay, as described in Materials and Methods. The comparative cytotoxicity is expressed as the 50% effective concentration (EC_{50}). Each value represents the mean±S.E. for three different experiments performed in triplicate.

RESULTS AND DISCUSSION

Naringenin (NGEN)-Induced Cytotoxicity in Various Human Cancer Cell Lines First, we examined the inhibitory effect of NGEN (10—2000 μM) in various human cancer cell lines (Table 1). In breast cancer, the estrogen receptor (ER) and progesterone receptor (PgR) are the most effective means by which to manage chemotherapy.\(^{11}\) NGEN has been reported to display strong estrogenic activity.\(^{12}\) There was no difference in the cytotoxicity of NGEN between human breast cancer cell line MCF-7, ER positive (+), PgR positive (+), and MDA-MB-231, ER negative (−), PgR negative (−) in this experiment. One of the most important genes regulating the effect of chemotherapy is p53.\(^{13}\) The p53 functions differently in wild-type (wt) and mutant (mt) cells.\(^{14}\) In fact, mutations of p53 in many human malignancies have been linked to a poor prognosis.\(^{15,16}\) We attempted to determine if p53 gene expression is involved in NGEN-induced cytotoxicity. The cytotoxicity was not changed by p53 in stomach (KATOIII and MKN-7) or liver (HepG2, Hep3B and Huh7) cancer cell lines. These results indicate that the mechanism of cytotoxicity by NGEN is independent of p53. In human cervical cancer cells (Hela and Hela-TG) was same level of cytotoxicity by NGEN. Gastric, pancreatic and colorectal cancers are major causes of morbidity and mortality worldwide. These malignancies tend to respond very poorly to chemotherapy and have a dismal prognosis.\(^{17}\) The inhibitory effect on cell growth by NGEN was observed not only in gastric cancer but also in human pancreas cancer (PK-1) or colon cancer (Caco-2). NGEN-induced cytotoxicity was low in Caco-2 compared to the other cell lines. However, HL-60 (promyelocytic leukemia), NALM-6 (B cell line of acute lymphocytic leukemia), Jurkat (T cell leukemia) and U937 (diffuse histiocytic lymphoma, expressing many monocyte-like characteristics) cells were more susceptible to NGEN. Anticancer drugs are generally more effective against leukemia than other malignancies, such as gastrointestinal cancer. These results suggest that NGEN is similar to other anticancer drugs.

NGEN-Induced Apoptosis in Human Colon Cancer Caco-2 Cells and Promyelocytic Leukemia HL-60 Cells Agents with the ability to induce apoptosis in tumors have the potential to be used for antitumor therapy. Flavonoids produce several biological effects, and apoptosis-inducing activities of flavonoids have been identified in several studies.\(^ {18}\) In order to elucidate the type of death induced by NGEN, apoptotic characteristics, including hypodiploidy (sub-G1 peak), of cells subjected to NGEN treatment were analyzed by flow cytometry using PI staining. As shown in Table 1, we decided to use the less susceptible Caco-2 cells and more susceptible HL-60 cells to examine the NGEN-induced cytotoxicity in this assay. NGEN dose-dependently induced apoptosis in both Caco-2 and HL-60 (Figs. 2A, B). The other cell lines also exhibited hypodiploidy on treatment with NGEN (data not shown). These results suggested that the cytotoxic activity of NGEN in Caco-2 and HL-60 cells occurs via apoptosis. Caco-2 cells required a higher concentration of NGEN than HL-60 cells to undergo apoptosis. These results correspond to the susceptibility of the cells to...
NGEN. However, a high concentration of NGEN (1000 μM) in HL-60 did not result in hypodiploid cells (data not shown). This indicates that a high concentration of NGEN causes necrosis not apoptosis.

**In Vivo Antitumor Effects of NGEN**

In order to demonstrate if NGEN has the ability to inhibit tumor growth in vivo, a study using ddY mice given a subcutaneous injection of sarcoma S-180 was performed. NGEN dose-dependently inhibited the growth of sarcoma S-180 when administered at 30, 100 or 300 mg/kg once a day for 5 d, with 99.7%, 72.2% or 57.0% suppression compared with the control (control tumor weight/H11005100%) following intraperitoneal (i.p.) injection, respectively (Fig. 3). The inhibitory effect on tumor growth by NGEN was exhibited after either intraperitoneal (i.p.) or peroral (p.o.) injection (54.1%). No significant toxicity was observed in NGEN-treated mice. The 50% lethal dose (LD50) of NGEN for 48 h was 15 g/kg (i.p.) in this experiment. 5-FU (i.p.), a typical anticancer drug, used as a positive control, showed 48.8% suppression in the control. Interestingly, naringin (NG) showed significantly suppression of tumor growth by peroral (p.o.) injection (57.8%) more than intraperitoneal (i.p.) injection (94.3%). This result indicates NGEN is active metabolite; orally administered NG was hydrolyzed by enterobacteria to NGEN before being absorbed.

NGEN is one of the flavonoids in citrus fruits. Flavonoids have been shown to have antitumor actions, causing the inactivation of carcinogen, cell cycle arrest, induction of apoptosis and inhibition of angiogenesis, antioxidation and the reversal of multidrug resistance or a combination of these mechanisms.19) Shen et al. reported that 2′-OH flavanone caused apoptosis and an increase in p21, but not p53 protein on subcutaneous injection in COLO205-induced tumors in nude mice and colorectal carcinoma cells.20) Our in vitro data partly supported that the NGEN-induced inhibitory effect on the growth of cancer cell lines was independent of p53 (Table 1). The mechanism of the inhibitory effect on tumor growth by NGEN has not been elucidated yet.

In conclusion, our results suggest that NGEN, one of the most abundant flavonoids in citrus fruits, has a potentially useful inhibitory effect on tumor growth. Medicine and one’s daily food are equally important in making a sick body well. NGEN is desirable to achieve an antitumor effect through internal means in cancer chemotherapy.

**REFERENCES**