Antiproliferative Effects of Isoflavones on Human Cancer Cell Lines Established from the Gastrointestinal Tract

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ABSTRACT

Seven isoflavones, biochanin A, daidzein, genistein, prunectin, and pseudobaptigenin were tested for cytostatic and cytotoxic effects on 10 newly established cancer cell lines of the human gastrointestinal origin. Proliferation of HSC-41E6, HSC-45M2, and SH101-P4 stomach cancer cell lines was strongly inhibited by biochanin A and genistein, whereas other stomach, esophageal, and colon cancer lines were moderately suppressed by both compounds. Biochanin A and genistein were cytostatic at low concentrations (<20 μg/ml for biochanin A, <10 μg/ml for genistein) and were cytotoxic at higher concentrations (>40 μg/ml for biochanin A, >20 μg/ml for genistein). DNA fragmentation was observed at cytotoxic doses of both compounds, indicating the apoptotic mode of cell death by the compounds. Chromatid condensation and nuclear fragmentation of each cell line were also observed. The advent of apoptosis was dose dependent for both isoflavones. Biochanin A suppressed tumor growth of HSC-45M2 and HSC-41E6 lines in athymic nude mice.

Our results suggest that two of isoflavone derivatives, biochanin A and genistein, inhibit the cell growth of stomach cancer cell lines in vitro through activation of a signal transduction pathway for apoptosis. Moreover, in vivo experiments demonstrate that biochanin A can be used as an anticancer agent.

INTRODUCTION

Although the prevalence of human gastric carcinoma is gradually decreasing recently, it still shares a significant portion of cancer-related deaths in Japan (1). In oriental societies, miso and soy sauce have been traditionally used in a daily diet. Ingestion of miso diet was shown to reduce the frequency of stomach cancers significantly as revealed in epidemiological studies (2). However, there is no experimental study on the mechanism of reduction in stomach cancer-related death among miso consumers. Miso is made by fermenting soybean, rice, and wheat or oat, and contains various biologically active substances such as botanic proteins, vitamins, fats, enzymes, carbohydrates, saponins, isoflavones, phytosterols, and lectins (3, 4). Among these, isoflavones are known to have various biological activities (4).

In the present study, we have tested the effect of isoflavones on human gastric tumor cell lines. For this, we have newly established and characterized six cancer cell lines derived from the human gastrointestinal tract. In addition, stomach cancer cell lines we have previously established were also included in the study (5, 6). The effects of seven different isoflavone compounds were examined on cell proliferation of these cell lines. The results indicated that some isoflavones induced apoptosis of tumor cells. In addition, these isoflavones suppressed tumor growth of the cell lines in athymic nude mice.

MATERIALS AND METHODS

Establishment of Cancer Cell Lines. Tumor tissues were trimmed of fat and necrotic portions and minced with scalpels. The tissue pieces were transferred, together with medium, at 10 to 15 fragments/dish to 60-mm culture dishes (Falcon, Lincoln Park, NJ). The patient’s ascitic tumor cells were harvested by centrifugation (1000 rpm for 10 min) and plated into 60-mm dishes. Culture dishes initially selectively trypsinized (trypsin, 0.05% w/v; EDTA, 0.02% w/v), to remove overgrowing fibroblasts. In addition, we attempted to remove the fibroblasts mechanically and to transfer the tumor cells selectively. The cells were cultured on dishes at 37°C in a 5% CO2 and 95% air atmosphere. In the early phase of cultivation, the cancer cells were grown in DMEM/α-minimal essential medium (1:1). When the cancer cells started to grow and become serially passageable, they were cultivated in DMEM (GIBCO, Grand Island, NY). Both media were supplemented with 10% heat inactivated FCS (Hazleton, Lenexa, KA), penicillin (100 IU/ml), streptomycin (100 μg/ml), 1% (v/v) nonessential amino acids, sodium pyruvate, and l-glutamine (2 mM). All cell lines were frozen in liquid nitrogen at early passage.

HSC-45 cell line was established from stomach cancer of the signet ring cell type from the ascites of a 28-year-old female patient. HSC-41 cell line was established from stomach cancer of the moderately differentiated tubular adenocarcinoma type from freshly resected surgical specimen of the primary tumor of a 45-year-old male patient. HSC-42 cell line was established from a xenotransplantable human stomach cancer line of the well-differentiated type, H-111 (7). HEC-46 cell line was established from a xenotransplantable human esophageal cancer line of the well-differentiated squamous cell carcinoma type, EH-68 (8). HCC-48 and HCC-50 cell lines were established from xenotransplantable human colorectal cancer lines of the well-differentiated adenocarcinoma type, CH-4 and CH-5, respectively (8).

HSC-39, HSC-40A, and HSC-43 cell lines were those established previously in our laboratory from signet ring cell gastric carcinomas with a scirrhous stromal reaction (5, 6), SH101 cell line was also reported previously, which was established from a stomach cancer of the well-differentiated adenocarcinoma type (8). ST-Fib and ST-Fib2 cells are normal fibroblasts from non-neoplastic mucosas of the human stomach (5). Subclones were obtained after two cycles of a metal cylinder isolation of colonies and subsequent colony formation in soft agar. Mycoplasma contamination was routinely monitored by a Hoechst 33258 staining kit (Flow Laboratories, McLean, VA).

Effect of Isoflavones on Cell Growth. Biochanin A and genistein were purchased from Sigma Chemical Co. (St. Louis, MO). Daidzein, genistin, prunectin, and pseudobaptigenin were obtained from Extrasynthese Laboratories (Genay, France). Puerarin was purchased from Funakoshi Chemicals Co., Ltd. (Tokyo, Japan). Isoflavone was dissolved in DMSO and aliquots were added to culture dishes. Final concentrations of DMSO in the culture medium were kept below 1% (v/v).

In order to determine the effect of various compounds on cell proliferation, cells were seeded at 2 × 104 cells with 1 ml of medium/well onto 24-well plates. On the indicated day thereafter, cells were trypsinized and the cell numbers were scored. The extent of growth was also determined by a crystal violet dye elution assay as described previously (9). An equivalent volume of DMSO was added to control cultures which usually had no measurable effects on cell growth.

Analysis of Apoptosis. For the detection of apoptosis, cultured cells were processed as described previously (10). Briefly, the cells were seeded onto tissue culture chamber slides (Nunc, Inc., Naperville, IL) and were incubated

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3 The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; TK, tyrosine kinase; ID50, drug concentration which inhibited growth by 50%.
with isoflavones of various concentrations for 48 h in 10% FCS plus DMEM. Cells were fixed with Carnoy's fixative and stained with Hecht meal 33258 fluorochrome. Photographs were taken under a Nikon EFD2 fluorescence microscope. Frequency of apoptotic cells was microscopically examined. The percentage of apoptotic cells in 1 × 10⁶ cells was calculated and data were expressed as mean ± SD (n = 3).

**Administration of Isoflavones in Tumor-bearing Athymic Nude Mice.** Six-week-old female BALB/c athymic nude mice were obtained from Charles River Japan Inc., Kanagawa, Japan. They were kept under sterile conditions in autoclaved cages with filter bonnets in laminar flow units and were fed sterilized MF pellets (Oriental Yeast Co., Ltd., Japan) and distilled water.

Biochanin A and genistein in 0.1 ml of DMSO were injected i.p. a day after cancer cell inoculation. Control animals were given the same vehicle. Six weeks later, mice were sacrificed and tumors were removed and weighed for size.

**RESULTS**

**Characterization of Newly Established Cancer Cell Lines.** Six human cancer cell lines (designated as HSC-41, HSC-42, HSC-45, HEC-46, HCC-48, and HCC-50) were newly established for the present study from tumors of the gastrointestinal tract. Subclones of 4 stomach cancer cell lines, HSC-39, HSC-40A(5), HSC-43(6), and SH101(8) were also included in the present study. Table 1 summarizes characteristics of these lines. All newly established cell lines were strictly anchorage independent and formed multilayered sheets with clusters upon confluence. HSC-45M2 and SH101-P4 cell lines were able to grow in chemically defined medium without any polypeptide growth factor.

All lines were tumorigenic in athymic BALB/c nude mice. Histologically, the transplanted tumors of HSC-41E6 and HSC-42H were classified as undifferentiated carcinomas composed of solid masses of tumor cells. HSC-45M2 cells formed signet ring cell carcinoma. Scirrhous stromal reactions noted in some of the original tumors were never observed in xenograft tumors. HEC-46R1 produced squamous cell carcinomas, resembling closely the original tumor. HCC-48B2 and HCC-50D3 cells formed well-differentiated adenocarcinomas.

Table 1 shows the secretion of the tumor-associated antigens, carcinoembryonic antigen, CA19-9, and tissue polypeptide antigen in vitro. HSC-45M2 cells secreted large amounts of carcinoembryonic and CA19-9 antigens. Production of these antigens were not detected in other newly established cell lines. Tissue polypeptide antigen was secreted from almost all the lines, and concentrations in the culture supernatant varied from 78 to >2000 units/ml.

**Effect of Isoflavones on Various Human Cancer Cell Lines.** The chemical structures of seven isoflavones, biochanin A, daidzein, genistein, genistin, prunectin, puerarin, and pseudobaptigenin were presented in Fig. 1. Effects of these isoflavones on cell proliferation were tested on 7 stomach cancer lines, 1 esophageal cancer line, 2 colon cancer lines, and on 2 normal stomach fibroblast lines. The cell growth was monitored by crystal violet dye elution assay 4 days after the addition of compounds. ID₅₀ was determined from the density of viable cells in cultures. Table 2 summarizes the results of the cell growth analysis.

Addition of biochanin A strongly inhibited proliferation of all cancer cell lines and two normal fibroblasts (Table 2; Fig. 1A). The ID₅₀ for the cancer cells varied from 7.6 to 17.7 μg/ml, whereas the ID₅₀ for the fibroblasts were 37 and 45 μg/ml, indicating that the latter was 3- to 5-fold resistant to biochanin A. Genistein was also a potent growth inhibitor on all cancer cell lines (ID₅₀, 6.8 to 15.5 μg/ml) and on normal fibroblast lines (ID₅₀, 20 and 25 μg/ml) (Table 2; Fig. 2B). Cancer cells and normal fibroblasts showed differential sensitivity to biochanin A and genistein. Daidzein was less effective and inhibited the growth of only two stomach cancer lines (HSC-45M2 and SH101-P4). The isoflavone β-glucoside, genistin, was not as effective and the ID₅₀ values were 18 μg/ml for HSC-45M2 cells and 33 μg/ml for HCC-48B2 cells. Although prunectin and pseudobaptigenin inhibited the growth of HSC-41E6 and HEC-46R1, and SH101-P4 and HCC-46R1 and HCC-50D3, the ID₅₀ values were very high, 500 μg/ml for HSC-45M2 and 250 μg/ml for HCC-48B2 and HCC-50D3 cells.

**Table 1. Biological characteristics of human cancer cell lines used in the study**

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<th>Cell line</th>
<th>Histological typing</th>
<th>Growth</th>
<th>DT (h)</th>
<th>Tumor-mor.</th>
<th>CEA (ng/ml)</th>
<th>CA19-9 (units/ml)</th>
<th>TPA (units/liter)</th>
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* Histological typing of the gastric carcinoma follow that of the Japanese Research Society for Gastric Cancer (1985). tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; sig, signet ring cell carcinoma; ud, undifferentiated carcinoma; sci, tumor with scirrhous stromal reaction; scc, squamous cell carcinoma; well, well-differentiated adenocarcinoma; A, ascitic tumor; P, primary tumor; X, xenotransplantable tumor; M, mono or multilayer; S, suspension; CDM, chemically defined medium supplemented with 0.05% bovine serum albumin. BT, doubling time of cells (determined as described in Ref. 12); ND, not done.

b Colony formation in semisolid agar was assayed as described (11).

c Viable cells (5 × 10^6) were injected s.c. into BALB/c athymic nude mice. Number of mice with tumors/number of mice given injection.

d Secretion of carcinoembryonic antigen (CEA), CA19-9, and tissue polypeptide antigen (TPA) was tested by radioimmunossay and immunoradiometric assay at Otsuka Assay Laboratories (Tokushima, Japan).

e Undetectable, below the range of detection of the assay.

f Cells were used as negative control.
INHIBITORY EFFECTS OF ISOFLAVONES ON CANCER CELL GROWTH

HO,T,,~O .,If ~ H3CO-~

Fig. 1. Chemical structures of the isoflavones. 1, biochanin A; 2, daidzein; 3, genistein; 4, genistin; 5, prunectin; 6, puerarin; 7, pseudobaptigenin.

48B2, respectively, both isoflavones showed no effect on other cell lines (Table 2). Puerarin, at a concentration as high as 80 μg/ml, was ineffective on any line tested here (Table 2; Fig. 2C).

These results indicated that three stomach cancer cell lines, HSC-41E6, HSC-45M2, and SH101-P4 were the most sensitive and responded in a dose-dependent manner to biochanin A and genistein. Other stomach, esophageal, and colon cancer cells were moderately sensitive to both isoflavones.

Growth Kinetics of Cells Treated with Biochanin A and Genistein. In order to study the cell kinetics in the inhibition in cell growth produced by biochanin A, cultures were exposed to 2.5-40 μg/ml of biochanin A in medium containing 10% FCS for 1 to 5 days. Biochanin A inhibited the cell proliferation of HSC-41E6, HSC-45M2, and SH101-P4 cells in dose- and time-dependent fashions (Fig. 3, A–C). High concentrations of the compound (>40 μg/ml) were cytotoxic to 3 cell lines upon continuous exposure for 4 days, resulting in >95% cell death as determined by trypan blue exclusion.

To ascertain the reversibility of the effect, cells were incubated with various concentrations of biochanin A for 48 h and the compound was removed thereafter (Fig. 3, E–G). At concentrations below 20 μg/ml, cells were able to resume growth after removal of the drug. In contrast, all stomach cancer cell lines failed to show regrowth at high doses of the compound (40 μg/ml) (Fig. 3 E–G).

Similar analysis was made on cells treated with genistein. Genistein inhibited the cell growth of SH101-P4 cells in dose- and time-dependent manners up to 20 μg/ml (Fig. 3D). At a concentration of less than 10 μg/ml of genistein, SH101-P4 cells showed regrowth after removal of the compound (Fig. 3H). In contrast, at 20 μg/ml and above, cells were unable to resume growth. HSC-41E6 and HSC-45 cells were inhibited similarly by genistein (not shown). Therefore, the growth inhibition at high doses of genistein was again irreversible.

Biochanin A and Genistein Induce Apoptosis. Irreversibility of growth inhibition by biochanin A and genistein may have been brought about by apoptotic cell death of human stomach cancer cells. Therefore, we examined the morphological changes of biochanin A-treated HSC-41E6, HSC-45M2, and SH101-P4 cells by Hoechst 33258 fluorochrome staining (Fig. 4). As shown in Fig. 4, C and F, chromatin condensation and fragmentation of nuclei were evident after a 48-h incubation with 40 μg/ml of biochanin A. Even at cytostatic doses such as 10 μg/ml, biochanin A induced nuclear fragmentation in HSC-41E6 (Fig. 4D), HSC-45M2 (Fig. 4E), and SH101-P4 (not shown). Such morphological changes were not observed in DMSO-treated control cells (Fig. 4, A and D).

Furthermore, fluorochrome staining of HSC-41E6, HSC-45M2, or SH101-P4 cells treated with 5, 10, and 20 μg/ml of genistein revealed extensive condensation of chromatin, fragmentation of nuclei, and the presence of debris (Fig. 4, H, I). In contrast, DMSO-treated control cells were normal and showed active growth with numerous mitoses (Fig. 4G).

DNA fragmentation was assessed in the HSC-41E6, HSC-45M2, and SH101-P4 cells after incubation with biochanin A and genistein

Table 2. Inhibition of growth of various human cancer cell lines by isoflavones

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<tr>
<th>Cell line</th>
<th>Biochanin A</th>
<th>Daidzein</th>
<th>Genistein</th>
<th>Genistin</th>
<th>Prunectin</th>
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a No observable effect, above the range of solubility of isoflavones in medium. Daidzein and genistein (≥40 μg/ml), prunectin and pseudobaptigenin (≥10 μg/ml), and puerarin (≥80 μg/ml).

b ND, not done.
Fig. 2. Effect of 3 isoflavones on the viability of various cancer cell lines. A, biochanin A; B, genistein; C, puerarin; O, HSC-45M2; ●, SH101-P4; □, HSC-41E6; ■, HCC-50D3; ▲, HEC-46R1; △, ST-Fib. The results of a representative experiment are given; points, average of 4 wells which varied by 10%. This experiment was done 3 times and gave similar results each time.

(Fig. 5). DNA recovered from cells treated for 48 h with biochanin A exhibited a “ladder” pattern of staining with integer multiples of roughly 180 base pairs (HSC-41E6; Fig. 5, Lanes 2–4; HSC-45M2; Fig. 5, Lanes 6–8, SH101-P4; not shown). A similar pattern of DNA fragmentation was observed in cells treated with genistein (SH101-P4; Fig. 5, Lanes 10–12, HSC-46E6 and HSC-45M2; not shown). These data confirmed that the morphological changes observed in Fig. 4 were indeed due to apoptosis.

In addition, the extent of apoptosis induced by biochanin A- or genistein-treated cell lines was dose dependent (Figs. 5 and 6).

Antineoplastic Effect of Biochanin A on HSC-45M2 and SH101-P4 Tumor Growth in Athymic Nude Mice. We tested the antineoplastic activity of biochanin A and genistein against human stomach cancer cells transplanted in BALB/c athymic nude mice. Biochanin A (4 mg/mouse) significantly inhibited tumor growth of HSC-45M2 and SH101-P4 (Table 3). At the end of the experiment, the mean tumor weight in the control and in the treated groups of HSC-45M2 was 570 ± 82 mg (ranging from 471 to 740 mg) and 310 ± 63 mg (ranging from 240 to 403 mg), respectively (P < 0.01). The tumor weight decreased to 54% in the biochanin A-treated group. In
INHIBITORY EFFECTS OF ISOFLAVONES ON CANCER CELL GROWTH

SH101-P4 tumor, the mean tumor weight in the control group was 2200 ± 190 mg (ranging from 2020 to 2400 mg), while that in the biochanin A-treated group was 850 ± 83 mg (ranging from 710 to 1011 mg) (P < 0.01). The weight in the biochanin A-treated group was only 38% of that of the control. Lower concentrations of biochanin A (0.5 and 1 mg) failed to suppress growth of both tumors (data not shown). The effect of biochanin A against these tumors was dose dependent. In contrast to HSC-45M2 and SH101-P4, tumor growth of HSC-41E6 was insensitive to biochanin A.

Administration of genistein at a dose of 4 mg/mouse did not show any effect on tumor growth of HSC-41E6, HSC-45M2, and SH101-P4 (Table 3). Changes in body weight were also examined in biochanin A- and genistein-treated mice. Body weight loss was not seen at these doses, neither were there any adverse effects. Mice administered biochanin A and genistein were histologically examined, and the liver, lung, heart, adrenal gland, kidney, and ovary were normal (data not shown).

Table 3: Effect of 2 isoflavones on HSC-41E6, HSC-45M2, and SH101-P4 tumor growth in BALB/c athymic nude mice

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<tr>
<th>Cells</th>
<th>Tumor wt (mg; mean ± SD)</th>
<th>Control</th>
<th>Biochanin A</th>
<th>Genistein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(DMSO) 2 mg 4 mg (4 mg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC-41E6</td>
<td>280 ± 31 (100) 300 ± 60 (107) 281 ± 93 (100) 297 ± 55 (106)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSC-45M2</td>
<td>570 ± 82 (100) 470 ± 65 (107) 310 ± 63 (100) 585 ± 90 (106)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SH101-P4</td>
<td>2220 ± 190 (100) 1390 ± 230 (103) 850 ± 83 (103) 1970 ± 250 (103)</td>
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<td></td>
</tr>
</tbody>
</table>

*a Viable cells (2 × 10⁶) were injected s.c. into 8-week-old BALB/c athymic nude mice. Biochanin A (200 μg or 400 μg/mouse/day) and genistein (400 μg/mouse/day) were administered i.p. daily 1 day after the inoculation for 10 times. Control animals received equal amounts of the vehicle.

*b Tumor weight was measured 6 weeks after inoculation of cells (n = 8).

**Numbers in parentheses, percentage of change; tumor weight of treated animal/that of control (=100%).

DISCUSSION

It is well known that soybean products in diet reduce the risk of cancer (3, 4). Therefore, we have examined the biological effect of the isoflavones contained in a soybean product, miso, on cell growth of newly established cancer cell lines. These isoflavones share a similarity in their chemical structures (Fig. 1). In the present study, we have shown that biochanin A or genistein strongly inhibited cell proliferation of stomach cancer cells, whereas other isoflavones required higher doses. Puerarin had no inhibitory activity on any lines examined. Analysis of growth kinetics of cells treated with biochanin A and genistein is summarized as follows: (a) dose- and time-dependent inhibition; (b) cytostatic activity at low concentrations; and (c) cytotoxic effect at high concentrations. Additionally, the inhibitory effect was pH dependent (data not shown).
An important feature of the cytotoxicity by biochanin A and genistein is that it is mediated through apoptosis. Apoptosis, known to be induced by a large variety of stimuli, including anti-cancer agents, differs from necrosis in many respects, the main difference being the active participation of cells in the process (13). We have found that apoptotic cells appeared not only at cytotoxic concentrations but also at cytostatic concentrations of the compounds in 3 stomach cancer cell lines. Furthermore, the advent of apoptotic features was dose dependent. It has been reported that genistein inhibits tyrosine kinase (14), DNA topoisomerase I and II (15), and ribosomal S6 kinase (16), and induces cytochrome P-450s (17).

We have recently reported induction of apoptotic cell death by transforming growth factor β1 in gastric carcinoma cells in vitro (10). In this system, both cell death and DNA fragmentation were partially inhibited by cycloheximide. However, cycloheximide was not effective for isofoavone-induced apoptosis (data not shown), suggesting the presence of a process not requiring protein synthesis. Our present results indicate that biochanin A and genistein inhibit growth of stomach cancer cells in vitro through activation of a signal transduction pathway for apoptosis.

DNA-damaging agents arrest cell cycle at G2 (18, 19). Recent studies have revealed that while daidzein, quercetin, flavone, and luteolin induced G1 arrest, genistein specifically blocked the cell cycle of human gastric cancer cells (HGC-27) at G2-M (20, 21). The transition from G2 to M is tightly linked to the kinase activity of the p34cdc2/cyclin B complex (22–24). Genistein might interfere directly or indirectly the phosphorylation state of the p34cdc2/cyclin complex which is responsible for controlling cell entrance into mitoses. Furthermore, it has been reported that interference of the cell cycle events by anticancer agents triggers an imbalance and leads to secondary changes, which are responsible for cell death (25).

No study was done on the mechanism of inhibition of cancer cells by biochanin A. Biochanin A has a phytoestrogenic activity (26). However, the extent of growth inhibition by biochanin A was similar for estrogen receptor-positive human breast cancer cell line MCF-7 cells and estrogen receptor-negative MDA-468 cells (27). This was taken to indicate that the isoflavones do not act via an estrogen receptor-dependent pathway. Further studies are required to elucidate the mechanisms.

Although growth of various tumor cells was inhibited by both compounds, growth of normal stomach fibroblasts was unaffected at the same concentration (Table 2; Fig. 2). Mechanism of this selectivity is not known at present. Genistein was shown to inhibit tyrosine kinases (TK) (14). Therefore, differences in the TK activity in each cell may explain the selectivity.

It was shown that TK inhibitors such as erbstatin and tyrophostins had an antineoplastic effect against human tumors of mammary, esophageal, and maxilla origins in xenograft systems (28, 29). We have examined the antineoplastic effect of biochanin A or genistein upon human stomach tumor-bearing athymic nude mice. Biochanin A (4 mg/mice) significantly inhibited HSC-45M2 and SH101-P4 tumor growth. Our data suggest that biochanin A may serve as an useful anticancer drug. However, growth of HSC-41E6 tumor was not suppressed by biochanin A, suggesting that action of this compound is cell type specific.

Our present study suggests that reduction of the risk of stomach cancer among miso consumers may partly be due to growth inhibition by isoflavones of gastric cancer cells.

ACKNOWLEDGMENTS

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