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Extract of Vinegar "Kurosu" from Unpolished Rice Inhibits the Proliferation of Human Cancer Cells

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The effects of the ethyl acetate extract of "Kurosu" (EK), Japanese traditional vinegar from unpolished rice, on the proliferation of a variety of human cancer cell lines were investigated by using the alamar blue assay. Cancer cell lines included colon adenocarcinoma (Caco-2), lung carcinoma (A549), breast adenocarcinoma (MCF-7), bladder carcinoma (5637), and prostate carcinoma (LNCaP) cells. EK inhibited the proliferation of all tested cell lines in a dose-dependent manner, with inhibition mostly pronounced in Caco-2 cells (up to 62% inhibition at a dose level of 0.025%). Flow cytometry of EK-treated Caco-2 cells showed a decrease in cell number in the G_2/M phase and an increase in the sub-G_1 phase (apoptotic). In addition, DNA fragmentation was detected in Caco-2 cells cultured with EK by immunostaining. RT-PCR analysis revealed p21 mRNA expression was induced in EK-treated Caco-2 cells. Moreover, PARP cleavage was promoted in EK-treated Caco-2 cells. These results suggest that EK causes G_0/G_1 arrest through p21 induction and, thus, is a potential apoptosis inducer in Caco-2 cells.

Key Words: "Kurosu", Vinegar, Human cancer cell line, Proliferation, Cell cycle, Apoptosis

Vinegar, which can be made from rice, apple, wine and various other substances, is a widely used acidic seasoning. One of the most common traditional vinegars in Japan, "Kurosu", is produced from unpolished rice, containing rice bran, through static surface acetic acid fermentation (1). "Kurosu" is characterized by the higher contents of amino acids and organic acids as compared to other vinegars. Physiologically, "Kurosu" has been shown to have various properties, including the prevention of hypertension in SHR rats (2).

We previously found the antioxidant activity of "Kurosu" (3), and isolated the active compounds, dihydroferulic acid (DFA) and dihydrosinapic acid (DSA), in "Kurosu". They are phenolic compounds related to ferulic and sinapic acids, respectively (4). In addition, we reported that an extract of "Kurosu" significantly suppressed 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced oxidative stress and tumor promotion (3). More recently, we found that an ethyl acetate extract of "Kurosu" (EK) inhibits colonic aberrant crypt foci (5), which are putative precursor lesions for colonic adenocarcinoma in rats (6), suggesting potential cancer chemopreventive substance against colon cancer. However, there are no reports on the effect of EK on human cancer cell lines.

In this study, we investigated the effect of EK on the growth of various human cancer cells. Since the frequency of malignancies in lung, colon, breast, and urinary bladder has been increasing in Japan, we chose human cancer cell lines, Caco-2 (a colon cancer cell line), A549 (a lung cancer cell line), MCF-7 (a breast cancer cell line), 5637 (a bladder cancer cell line), and LNCaP (a prostate cancer cell line) to examine antiproliferative effects of EK. In addition, we analyzed the effects of EK on cell cycle, apoptosis, and several proteins involved in cell cycle and apoptosis in Caco-2 cells.

Materials and Methods

Chemicals and cell lines. "Kurosu" was made by Tamanoi Vinegar Co., Ltd. (Nara, Japan). The Caco-2, 5637, A549, and MCF-7 cell lines were purchased from Riken Cell Bank (Ibaraki, Japan), and LNCaP cells were purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). RPMI 1640 medium, non-essential amino acid solution, and trypsin were purchased from Life Technologies (Tokyo, Japan). Eagle's minimum essential medium (MEM) and Dulbecco's

modified Eagle medium (DMEM) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Cansera International Inc. (Ontario, Canada). Amberlite XAD-4 was purchased from Organo Co., Ltd. (Tokyo, Japan).

EK. EK was prepared according to the method described previously (5). "Kurosu" was adsorbed on Amberlite XAD-4, and the resin was washed with ion-exchanged water to remove non-adsorbed material. The adsorbed fraction was eluted with methanol, and the solvent was evaporated under reduced pressure. The residue was extracted with ethyl acetate, and the solvent was evaporated under reduced pressure. The resulting EK (yield, 0.4 g/L "Kurosu") was dissolved in DMSO and used for assays.

Cell culture. Caco-2 cells were cultured in MEM (pH 7.4) supplemented with 20% FBS and 1% non-essential amino acid solution, while MCF-7 cells were cultured in the same medium, except that the concentration of FBS was 10%. A549 cells were cultured in DMEM (pH 7.4) supplemented with 10% FBS, while the 5637 and LNCaP cell lines were cultured in RPMI 1640 supplemented with 10% FBS. All cell lines were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Cell proliferation assay. Exponentially growing cells were seeded in 96-well cell culture plates and allowed to adhere for 24 hrs. The initial cell density of the A549 cells was 5 x 10^2/well and that of the other cells was 2 x 10^3/well. After 24 hrs, EK was added to the medium, at concentrations of 0%, 0.01%, 0.025%, 0.05%, 0.075% and 0.1%, and cells were allowed to grow for another 72 hrs except for the Caco-2 cell line, which was allowed to grow for 96 hrs after the addition of EK. Since EK was dissolved in DMSO, the solvent concentration was kept constant, at 0.2%, in all experiments. Cell counts were determined by a protocol using alamarBlue™ (Biosource International Inc., Camerillo, CA), which becomes fluorescent after reduction by the metabolic activity of living cells. After 3 hrs of incubation with alamarBlue™ at 37°C in a humidified 5% CO_2 atmosphere, cell counts were determined based on a calibration curve for each cell line. The calibration curve was generated using cell numbers between 5 x 10^2 and 1.6 x 10^5 cells, and adjusted after the determination of cell numbers with a hemocytometer. Fluorescence was measured at 590 nm after excitation at 544 nm using a fluorescence multi-well-plate reader (Fluoroskan Ascent FL, ThermoLabsystems, Vantaa, Finland). All analyses were performed on sets of 6 wells.

Flow cytometry. The effect of EK on Caco-2 cell proliferation was evaluated by measuring the distribution of the cells in the different phases of the cell cycle by flow cytometry. This determination was based on the measurement of the DNA content of cells labeled with propidium iodide. Cells (2.0 x 10^5 per 25-cm^2 culture flask) were incubated for 24 hrs. EK was added to each flask, to a final concentration of 0.025% and 0.05%, and the cells were further incubated. After 24 hrs, 48 hrs, 72 hrs, and 96 hrs, the cells were trypsinized and collected (5 x 10^5 cells/tube). The cells were fixed by suspending the pellet in cold 70% ethanol and stored at -20°C until use. The cells were subsequently centrifuged for 5 min, resuspended in Phosphate-Citrate Buffer (192 mM Na_2HPO_4, 4 mM citric acid), and incubated for 30 min at room temperature. After centrifugation for 5 min, the pellet was resuspended and incubated for 20 min in 10 µg/ml PI, 1 µg/ml RNase. Cell cycle analysis was performed using EPICS XL (Beckman Coulter, Inc., Fullerton, CA).

Immunocytochemistry. Caco-2 cells were seeded in 25-cm^2 culture flasks and incubated with EK as described above. After 100 hrs of incubation with EK, the cells were trypsinized, collected, prepared on slide glasses, and stored in 95% ethanol until use. To detect apoptotic cells, the slides were immunostained with rabbit polyclonal antibody to single stranded DNA (DakoCytomation, Kyoto, Japan), according to the manufacturer's instructions (7, 8).

RT-PCR. Caco-2 cells, incubated for 24, 48, and 72 hrs with EK, were trypsinized and collected (2 x 10^6 cells/tube). Total RNA was purified with Isogen (Nippon Gene, Tokyo, Japan) and reverse transcribed to cDNA. PCR was performed using the oligonucleotide primers as follows; p21, (F) 5'-AAAGGCCCGCTCTACATCTT-3' and (R) 5'-ACAAGTGGGGAGGAGGAAGT-3'; GADD45, (F) 5'-TGCCATTATCTCAAAGGTGA-3' and (R) 5'-GAAGCCTGCTTATTCGCAGTA-3'; GAPDH, (F) 5'-CGAGATCCCTCCAAAATCAA-3' and (R) 5'-AGGTCCACCACTGACACGTT-3'.

Western blotting. Caco-2 cells, incubated for 24, 48, and 72 hrs with EK, were trypsinized and collected (2 x 10^6 cells/tube). The cells were resuspended in RIPA

buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and centrifuged at 1,300 rpm for 5 min. The protein concentrations of the supernatants were determined using a protein assay kit (Pierce Biotechnology, Rockford, IL). Proteins (20 μg) were separated by SDS polyacrylamide gels electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with a mouse monoclonal antibody to caspase-3 (Transduction laboratories, Lexington, KY) or a rabbit polyclonal antibody to PARP (Cell Signaling Technology, Inc., Beverly, MA) and then with the appropriate secondary antibody (sheep polyclonal anti-mouse IgG for caspase-3 and donkey anti-rabbit IgG for PARP; Amersham Pharmacia Biotech, Piscataway, NJ). The blots were developed using an ECL detection kit (Amersham Pharmacia Biotech).

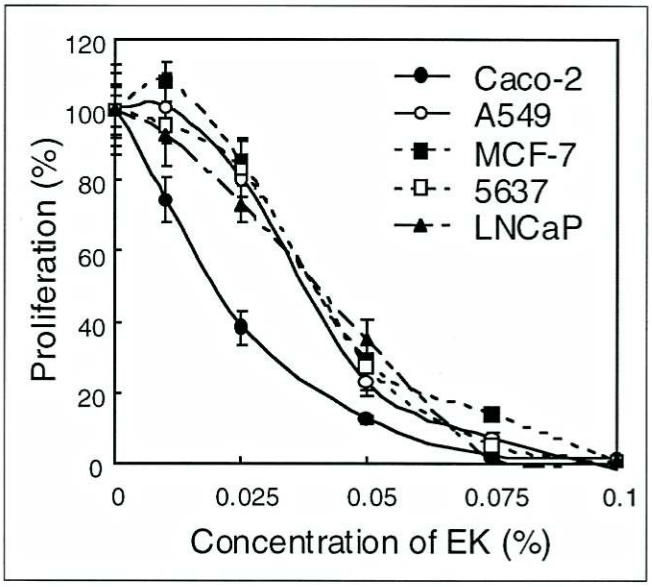

Fig. 1 - Dose response of human cancer cell lines to EK. The five types of human cancer cell lines, Caco-2 (●), MCF-7 (■), 5637 (□), and LNCaP (▲), were plated at a density of $2x10^3$ cells/well, while A549 (○) cells were plated at $5x10^2$ cells/well, in 96-well plates as described in "Materials and methods", and allowed to attach overnight. Subsequently, six wells each were treated with each dose of EK, ranging from 0% to 0.1%. After 72 hrs (96 hrs for Caco-2), the number of viable cells per well was determined by the alamar blue assay. Each point represents mean ± SD. Dose-dependent inhibition by EK on growth were observed in all five cell lines.

Results

Effects of EK on proliferation of human cancer cells. Treatment of five human cancer cell lines with EK resulted in dose-dependent inhibition of growth of each (Fig.1), with growth inhibition of Caco-2 cells most pronounced of the five cell lines tested. Relative to their growth in the absence of EK, treatment of Caco-2 cells with 0.025% and 0.05% EK resulted in 38.1% ($p < 0.001$) and 12.3% ($p < 0.001$) growth, respectively. Treatment of the four other cancer cell lines, A549, MCF-7, 5637, and LNCaP, with 0.025% EK resulted in growth between 73.5% and 82.5%, while treatment with 0.05% EK resulted in growth between 22.9% and 34.8%, relative to growth in the absence of EK. In addition, Caco-2 cells treated with EK showed morphological changes suggestive of apoptosis, including the formation of apoptotic bodies (data not shown).

EK alters the cell cycle distribution in Caco-2 cells. When Caco-2 cells were exposed to 0.025% EK, at which concentration proliferation was inhibited by 61.9%, flow cytometry analysis showed a time-dependent reduction in the number of cells in the G_2/M phase of the cell cycle (Fig. 2a). In cells incubated with 0.05% EK, there was a 10% increase in the number of cells in the G_0/G_1 phase and a decrease in the G_2/M phase from 0 to 72-h exposures. Subsequently the number of cells in the G_0/G_1 phase decreased about 15%, and there was an increase in the number of cells in the sub-G_1 phase, which correlated with enhanced DNA fragmentation (Fig. 2b). These results suggest that EK causes G_0/G_1 cell cycle arrest and induces apoptosis in Caco-2.

EK increases ssDNA in Caco-2 cells. Immunocytochemistry revealed that there were positive cells for ssDNA when Caco-2 cells cultured in the presence of 0.025% EK for 100 hrs were stained with the antibody against single-stranded DNA (Fig.3).

Effect of EK on expression of p21 and GADD45 mRNA. RT-PCR analysis revealed that, when Caco-2 cells were incubated with 0.05% EK, there was a time-dependent increase in p21 mRNA expression (Fig.4). GADD45 mRNA expression was not affected by exposure to EK (Fig.4).

Effect of EK on expression of Caspase-3 and PARP proteins. Western-blotting analysis showed that full-length PARP (116 kDa) expression was increased time-dependently in Caco-2 cells exposed to 0.05% EK

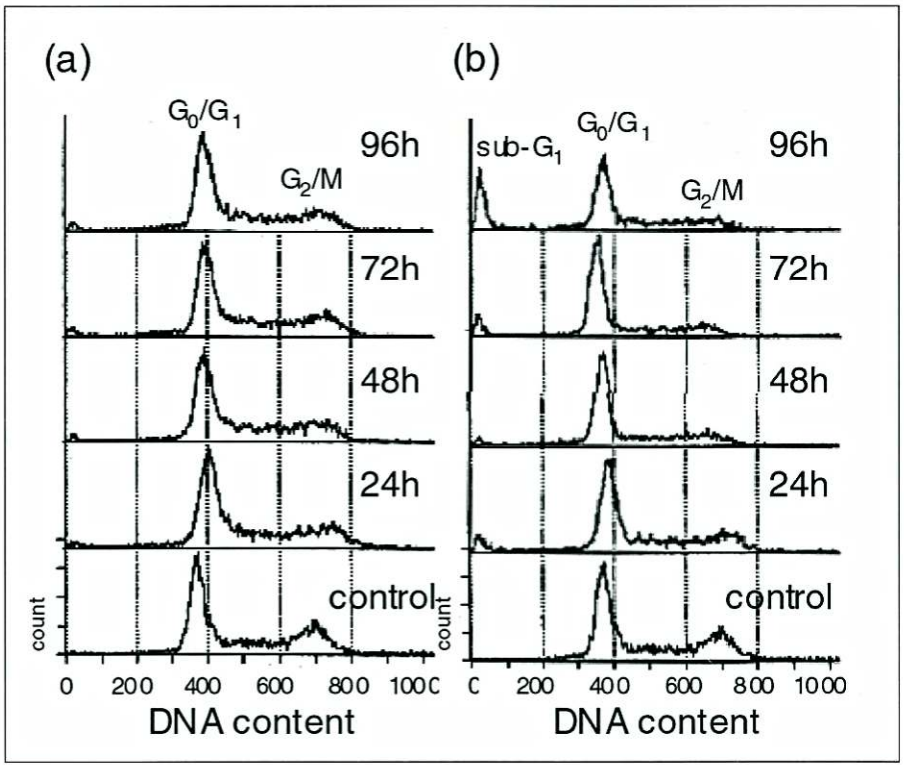

Fig. 2 - DNA content of Caco-2 cells cultured with EK. Caco-2 cells were plated at a density of $2.0x10^5$ cells per 25-cm^2 culture flask and allowed to attach overnight. Cells were cultured with (a) 0.025% or (b) 0.05% EK for 24, 48, 72, or 96 hrs, harvested, stained with propidium iodide, and analyzed for DNA content by fluorescence-activated cell sorter analysis.

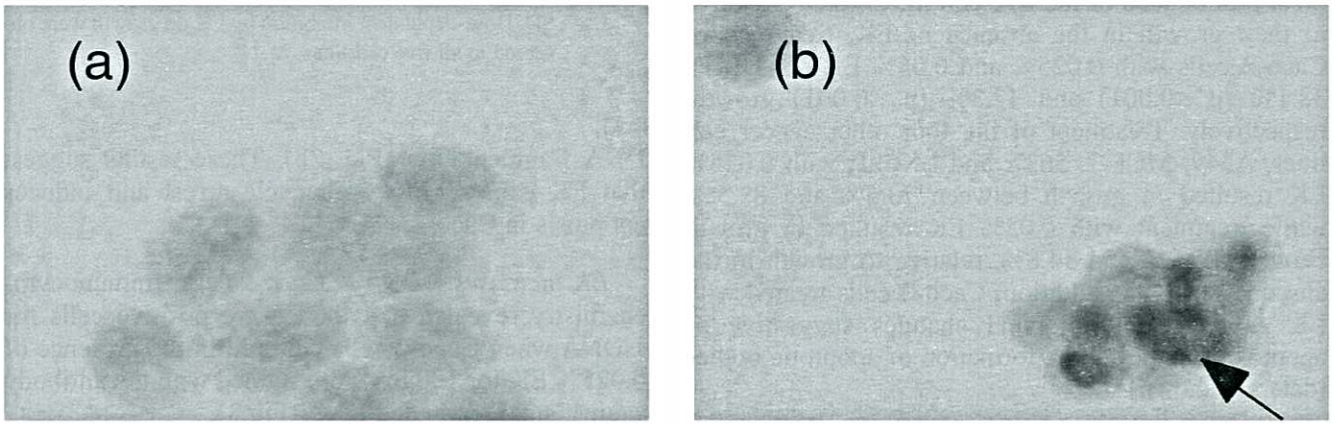

Fig. 3 - Immunocytochemistry of Caco-2 cells with rabbit polyclonal anti-single stranded DNA antibody. Caco-2 cells ($2.0x10^5$ per 25-cm^2 culture flask) were allowed to attach overnight and treated with (a) 0% or (b) 0.025% EK for 100 hrs. Cells were harvested, fixed, and stained with rabbit polyclonal antibody against ssDNA. Arrow indicates ssDNA. Original magnification, x 40.

(Fig.5). Cleaved PARP (89 kDa) was also detected after exposure of the cells for 48 hrs and 72 hrs. There was no change in the expression level of pro-caspase-3 after exposure to EK (Fig.5). Ponceau S staining for total protein on each immunoblot confirmed equal loading and transfer of proteins among lanes (data not shown).

Discussion

We have shown here that addition of EK to the culture medium induced dose-dependent suppression of proliferation of all five human cancer cell lines tested. It is of interest that EK exerts inhibitory effects on a wide variety of cancer cell types, including those

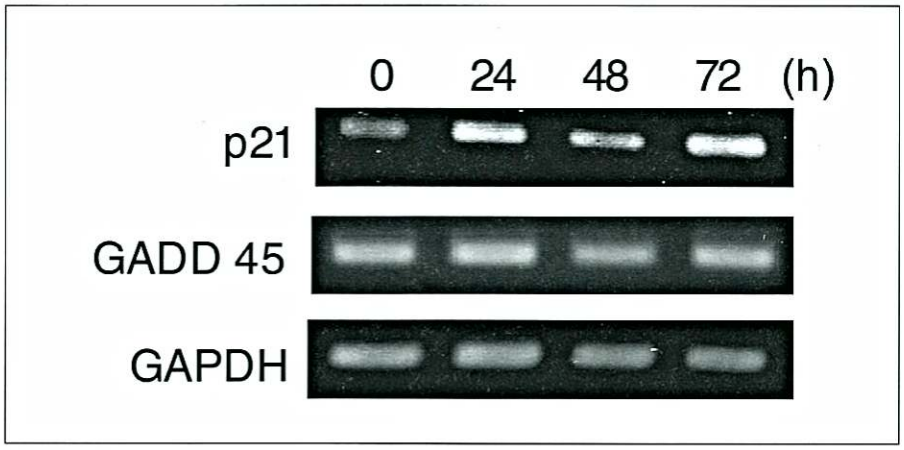

Fig. 4 - Effect of EK on expression of p21 and GADD45 mRNA in Caco-2 cells. Cells were treated with 0.05% EK for 24, 48, or 72 hrs, and p21 and GADD45 mRNA levels were determined by RT-PCR. The GAPDH mRNA was used to normalize the amounts of RNA loaded onto each lane.

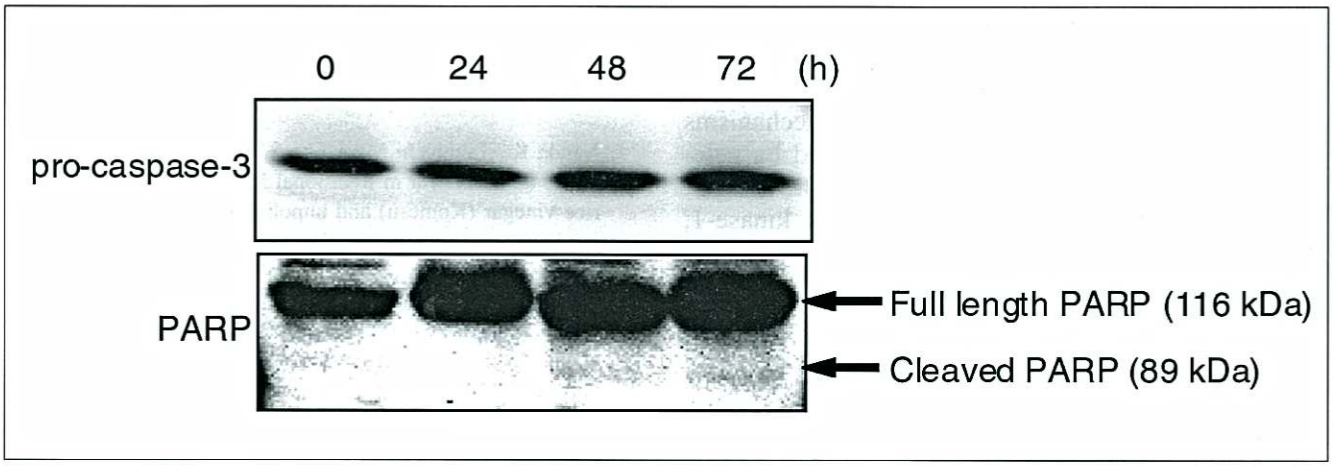

Fig. 5 - Effect of EK on expression of PARP and pro-caspase-3 in Caco-2 cells. Cells were treated with 0.05% EK for 24, 48, or 72 hrs, and pro-caspase-3 and PARP protein levels were determined by Western blot analysis.

derived from colon, lung, breast, bladder, and prostate tumors. It is likely that EK has a potential cancer inhibitory action in these organs, although the chemical(s) responsible for the effects in EK are not yet known.

EK most effectively inhibited the growth of Caco-2 cells, inducing G_0/G_1 arrest and apoptosis. RT-PCR analysis revealed that EK elevated the expression of p21 mRNA, while Western analysis showed that EK promoted PARP cleavage, in Caco-2 cells. These results suggest that EK causes G_0/G_1 arrest through p21 induction, and may induce apoptosis in these cells.

"Kurosu", a traditional health food in Japan, possesses antioxidative activity (3) and contains the ferulic acid derivatives, DFA and DSA (4). Phenolic compounds in ethyl acetate extract of brown rice have been shown to inhibit the growth of human breast and colon cancer cells (9). "Kurosu" is made from unpolished rice with bran (i.e. brown rice) and has higher amounts of phenolic compounds, including DFA, than rice vinegar from polished rice (3, 4). These phenolic compounds, which are probably generated from rice bran in "Kurosu", as well as other components generated during its fermentation, may be responsible for the inhibitory effects of EK on human cancer cell proliferation.

We have recently found that EK effectively inhibited the development of AOM-induced colonic ACF in rats and decreased proliferating cell nuclear antigen-labeling indices in ACF (5). These results suggest that EK may inhibit the initiation or the proliferation of initiated cells *in vivo* as well as EK inhibits the proliferation of cancer cells *in vitro*.

Flow-cytometoric analysis revealed that EK can arrest Caco-2 cells in the G_0/G_1 phase, prior to the occurrence of apoptosis. The antiproliferative effect of

EK on Caco-2 cells seemed possibly to occur via induction of cell cycle arrest at G_0/G_1 and apoptosis as did isoprenoid (10) and cyclooxygenase-2 (COX-2) inhibitor (11).

In a previous study, we showed that EK has anti-inflammatory effects on mouse skin (3). Moreover, we found that EK inhibits the expression of COX-2 proteins in RAW264.7 cells treated with lipopolysaccharide and interferon-γ (unpublished data). Several studies have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitors suppress cancer cell proliferation (11-13). Thus, like the NSAIDs, EK may act to modulate carcinogenesis secondary to inflammation.

The selective COX-2 inhibitor celecoxib has been reported to induce cell cycle arrest and apoptosis in colon cancer cell lines. Since these effects are associated with different levels of expression of COX-2, it is likely that the antitumor effects of celecoxib probably are mediated through COX-2 independent mechanisms (11). The cell cycle arrest at G_0/G_1 induced by celecoxib may thus be due to a decreased expression of cyclin A, cyclin B1, and cyclin-dependent kinase-1, and/or to an increased expression of the cell cycle inhibitory proteins, $p21^{Waf1}$ and $p27^{Kip1}$ (11). In the present study, EK suppressed the proliferation of human cancer cell lines that express COX-2 (Caco-2, A549 and 5637) as well as lines that do not express this enzyme (MCF-7 and LNCaP) (14-18). This suggests that, like celecoxib, EK may alter the expression of cyclin and cell cycle inhibitory proteins, at least in part through COX-2 independent mechanisms. As expected, RT-PCR analysis revealed that EK increased the mRNA encoding the cell cycle inhibitory protein, p21, suggesting that EK may induce G_0/G_1 cell cycle arrest by inducing p21 expression.

Western blotting analysis indicated that EK also increases the expression and cleavage of PARP. Transient activation of PARP has been reported to occur early in apoptosis, followed by its degradation by caspase-3-like protease (19). PARP-1 activation has recently reported to trigger release of a mitochondrial proapoptotic protein called apoptosis-inducing factor (AIF), which promotes programmed cell death through a caspase-independent pathway (20). These results taken together suggest that EK may trigger the apoptotic process by increasing the level of PARP and its subsequent degradation.

Although caspase-3 is the major protease responsible for the *in vivo* cleavage of PARP, other caspases, such as caspase-7, have also been shown to partially cleave PARP at much lower efficiencies (19, 21). In the present study, reduction of pro-caspase-3 level is not observed, suggesting that EK may activate caspase-3 below the detection limit of the method we used, or that it may activate other caspases that cleave PARP.

In summary, EK derived from the traditional Japanese vinegar "Kurosu" inhibited the growth of human cancer cell lines, possibly through induction of cell cycle arrest and apoptosis. These findings suggest that EK may have a potential for cancer prevention, although further studies are needed to determine the chemical constituents in EK responsible for the induction of cell cycle arrest and apoptosis.

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References

1. Nanda K., Taniguchi M., Ujike S. et al.: Characterization of acetic acid bacteria in traditional acetic acid fermentation of rice vinegar (Komesu) and unpolished rice vinegar (Kurosu) produced in Japan. Appl. Environ. Microbiol. 67: 986-990, 2001.
2. Nishikawa Y., Takata Y., Nagai Y., Mori T., Kawada T., Ishihara N.: Antihypertensive effect of Kurosu extract, a traditional vinegar produced from unpolished rice, in the SHR rats. Nippon Syokuhin Kagaku Kogaku Kaishi (in Japanese). 48: 73-75, 2001.
3. Nishidai S., Nakamura Y., Torikai K. et al.: Kurosu, a traditional vinegar produced from unpolished rice, suppresses lipid peroxidation *in vitro* and in mouse skin. Biosci. Biotechnol. Biochem. 64: 1909-1914, 2000.
4. Shimoji Y., Tamura Y., Nakamura Y. et al.: Isolation and identification of DPPH radical scavenging compounds in Kurosu (Japanese unpolished rice vinegar). J. Agric. Food Chem. 50: 6501-6503, 2002.
5. Shimoji Y., Sugie S., Kohno H. et al.: Extract of vinegar "Kurosu" from unpolished rice inhibits the development of colonic aberrant crypt foci induced by azoxymethane. J. Exp. Clin. Cancer Res. 22(4):591-598, 2003.
6. Bird R.P.: Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett. 93: 55-71, 1995.
7. Naruse I., Keino H., Kawarada Y.: Antibody against single-stranded DNA detects both programmed cell death and drug-induced apoptosis. Histochemistry 101: 73-78, 1994.
8. Watanabe I., Toyoda M., Okuda J. et al.: Detection of apoptotic cells in human colorectal cancer by two different *in situ* methods: Antibody against single-stranded DNA and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) methods. Jpn. J. Cancer Res. 90: 188-193, 1999.
9. Hudson E.A., Dinh P.A., Kokubun T., Simmonds M.S.J., Gescher A.: Characterization of potentially chemopreventive

phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. Cancer Epidemiol. Biomarkers Prev. 9: 1163-1170, 2000.
10. Mo H., Elson C.E.: Apoptosis and cell-cycle arrest in human and murine tumor cells are initiated by isoprenoids. J. Nutr. 129: 804-813, 1999.
11. Grösch S., Tegeder I., Niederberger E., Bräutigam L., Geisslinger G.: COX-2 independent induction of cell cycle arrest and apoptosis in colon cancer cells by the selective COX-2 inhibitor celecoxib. FASEB J. 15: 2742-2744, 2001.
12. Shiff S.J., Koutsos M.I., Qiao L., Rigas B.: Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocarcinoma cells: Effects on cell cycle and apoptosis. Exp. Cell Res. 222: 179-188, 1996.
13. Xu X.C.: COX-2 inhibitors in cancer treatment and prevention, a recent development. Anticancer Drugs 13: 127-137, 2002.
14. Tsuji S., Kawano S., Sawaoka H. et al.: Evidences for involvement of cyclooxygenase-2 in proliferation of two gastrointestinal cancer cell lines. Prostaglandins Leukot. Essent. Fatty Acids 55: 179-183, 1996.
15. Heasley L.E., Thaler S., Nicks M., Price B., Skorecki K., Nomenoff R.A.: Induction of cytosolic phospholipase A_2 by oncogenic Ras in human non-small cell lung cancer. J. Biol. Chem. 272: 14501-14504, 1997.
16. Bostrom P.J., Aaltonen V., Soderstom K.O., Uotila P., Laato M.: Expression of cyclooxygenase-1 and -2 in urinary bladder carcinomas *in vivo* and *in vitro* and prostaglandin E2 synthesis in cultured bladder cancer cells. Pathology 33: 469-474, 2001.
17. Liu X.H., Rose D.P.: Differential expression and regulation of cyclooxygenase-1 and -2 in two human breast cancer cell lines. Cancer Res. 56: 5125-5127, 1996.
18. Zha S., Gage W.R., Sauvageot J. et al.: Cyclooxygenase-2 is up-regulated in proliferative inflammatory atrophy of the prostate, but not in prostate carcinoma. Cancer Res. 61: 8617-8623, 2001.
19. Simbulan-Rosenthal C.M., Rosenthal D.S., Iyer S., Boulares A.H., Smulson M.E.: Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. J. Biol. Chem. 273: 13703-13712, 1998.
20. Yu S.W., Wang H., Poitras M.F. et al.: Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297: 259-263, 2002.
21. Cohen G.M.: Caspases: the executioners of apoptosis. Biochem. J. 326:1-16, 1997.

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