Fucoidan, a major component of brown seaweed, prohibits the growth of human cancer cell lines *in vitro*

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Abstract. Fucoidan, the general term for sulfated polysaccharides, is reported to engage in various biological activities having anti-tumor, anti-coagulation and anti-viral effects. Though it has been investigated, the mechanism of its anti-tumor effects remains elusive. The current study examined the anti-tumor effects of fucoidan extracted from Okinawa mozuku on 15 human cancer cell lines (6 hepatocellular carcinomas, 1 cholangiocarcinoma, 1 gallbladder cancer, 2 ovarian cancers, 1 hepatoblastoma, 1 neuroblastoma and 3 renal cancers) using an MTT assay. Changes in apoptosis and the cell cycle were analyzed by flow cytometry. The results revealed that cell proliferation was suppressed in 13 cell lines in a time- and/or dose-dependent manner; this suppression was marked in the hepatocellular carcinoma, cholangiocarcinoma and gallbladder carcinoma cell lines. In contrast, proliferation of the neuroblastoma and 1 of the 2 ovarian carcinoma cell lines was not affected. The ratio of apoptotic cells significantly increased in 5 of the 6 hepatocellular carcinoma cell lines, and the ratio of G_2/M cells increased in the 3 hepatocellular cell lines examined. These observations indicate that fucoidan is a potential anti-tumor agent for the treatment of bile duct cancers, such as hepatocellular carcinoma, cholangiocarcinoma and gall-bladder carcinoma.

Introduction

Malignant tumors are a major cause of death in humans and, though treatment methods for cancer have markedly progressed, curative regimens and protocols are still under investigation. At present, chemotherapy is regarded as the most effective treatment for solid tumors.

Recent studies have shown that the natural substances extracted from green tea (1) and marine products, among others, have favorable preventive effects against cancers. 'Mozuku' (brown seaweed) is a marine plant which grows offshore around Okinawa Island. It has attracted the attention of scientists as well as the general public, and is considered a food which has various beneficial effects on the body. Fucoidan, the major component of 'mozuku', is the general term used for sulfated polysaccharides, which are also found in other seaweeds such as 'wakame', 'hijiki', 'mekabu'. Sulfated polysaccharides are reported to engage in various biological activities having anti-tumor effects (2-6), anti-thrombin activity (7,8), anti-coagulant activity (9) and anti-viral effects (10,11). Their anti-tumor effects have been investigated using various methods, but the mechanism of their action has remained elusive.

In order to increase the understanding of these anti-tumor effects, this *in vitro* study was conducted using 15 cell lines from 5 types of human cancers (liver cancer, cholangiocarcinoma, ovarian cancer, hepatoblastoma and neuroblastoma) along with fucoidan extracted from 'Okinawa mozuku'.

Materials and methods

Preparation of culture medium with fucoidan. Fucoidan solution was kindly provided by FCC Horiuchi & Co. (Kurume, Japan). The fucoidan was extracted from mozuku (*Cladosiphon Okamuranus Tokida*) collected from the shores of Okinawa Island in Japan, sold as a sea product (containing fucose 22.1 mg/100 mg) with government approval, and used as raw material for a health drink. The structure of the polysaccharide from *C. okamuranus* has been investigated previously (12,13).

The basal medium for cell culture was Dulbecco's modified Eagle's medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 5% fetal bovine serum (Whittaker Bioproducts Inc., Walkersville, MD), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco, Chagrin Falls, OH). Fucoidan was diluted with this medium and prepared in 10 concentrations (0.35, 0.70, 1.40, 2.82, 5.63, 11.25, 22.50, 45, 90 and 180 μg/ml). The osmotic pressures and pH values of the cultures with or without fucoidan were within normal physiological range.

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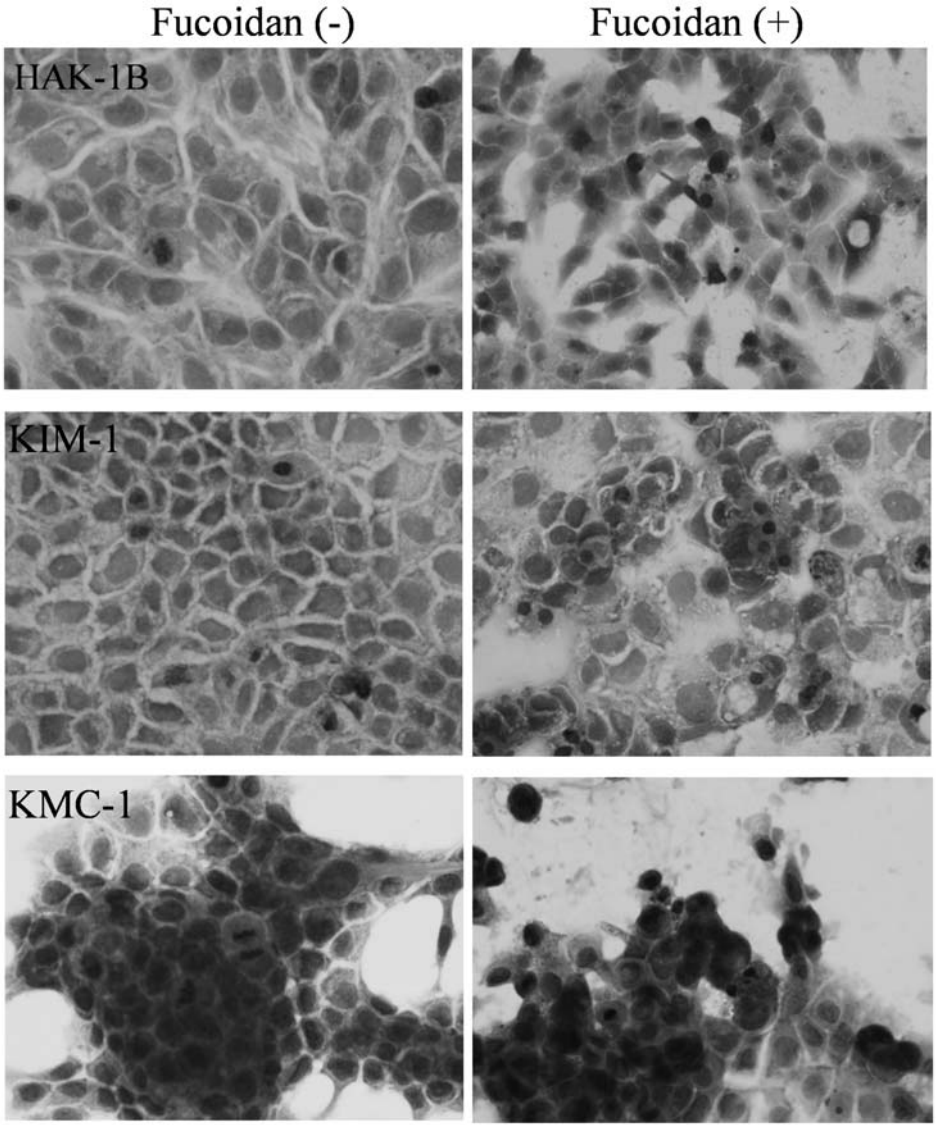

Figure 1. Photomicrograph of HAK-1B (A and B), KIM-1 (C and D) and KMC-1 (E and F) cells cultured for 72 h on a Lab-Tek Chamber slide. (A, C and E) No fucoidan in culture medium. Some mitotic figures were noted. (B, D and F) With 22.5 μg of fucoidan in culture medium. Apoptotic cells were characterized by cytoplasmic shrinkage, chromatic condensation and nuclear fragmentation (H&E staining, x200).

Cell lines and cell culture. Fifteen cell lines were used. These included 6 human hepatocellular carcinoma (HCC) cell lines [KIM-1 (12,14), KYN-1 (15), KYN-2 (16), KYN-3 (17), HAK-1A, HAK-1 (18)], one cholangiocarcinoma cell line (KMC-1) (19) and one gallbladder carcinoma cell line (KMG-C) which were originally established in our laboratory. Two human ovarian clear cell carcinoma cell lines (KOC-5C and KOC-7C) were established as described elsewhere (20,21), as were 3 human renal cell carcinoma cell lines (KURU II, KURM and OSRC2) (22). The human neuroblastoma cell line (SK-N-SE) was a generous gift from Dr K. Ueda of the Department of Pediatrics and Child Health of Kurume University. The human hepatoblastoma cell line (HuH-6) was purchased from the Japan Health Sciences Foundation (Osaka, Japan).

Observation of morphological changes. For light microscopic observations, the cells were seeded on Lab-Tek Tissue Culture Chamber Slides (Nunc Inc., Roskilde, Denmark), cultured with or without fucoidan (2.82, 22.5 or 90 μg/ml) for 72 h, fixed in Carnoy's solution for 10 min, then stained with hematoxylin and eosin (H&E) and observed under a microscope (Olympus BH-2, Olympus Optical, Tokyo, Japan).

Effect of fucoidan on the proliferation of each cell line. The effect of fucoidan on cell proliferation was investigated with colorimetric assays using MTT [3-(4,5-dimethylthiazol-2yl-yl)-2,5-diphenyltetrazolium bromide] cell growth assay kits (Chemicon International Inc., Temecula, CA) as previously described (23). Briefly, cells were seeded on 96-well plates (Falcon, Becton Dickinson Labware, Tokyo, Japan) and cultured for 24 h. The medium was then replaced with 100 μl of fresh medium alone or containing the diluted fucoidan solution (0.35, 0.70, 1.40, 2.82, 5.63, 11.25, 22.50, 45, 90 or 180 μg/ml). After 24, 48, 72 or 96 h, the number of viable cells was counted. Each experiment was repeated at least twice. The 50% inhibitory concentration (IC_{50}) was defined as the fucoidan concentration (μg) that caused a 50% reduction

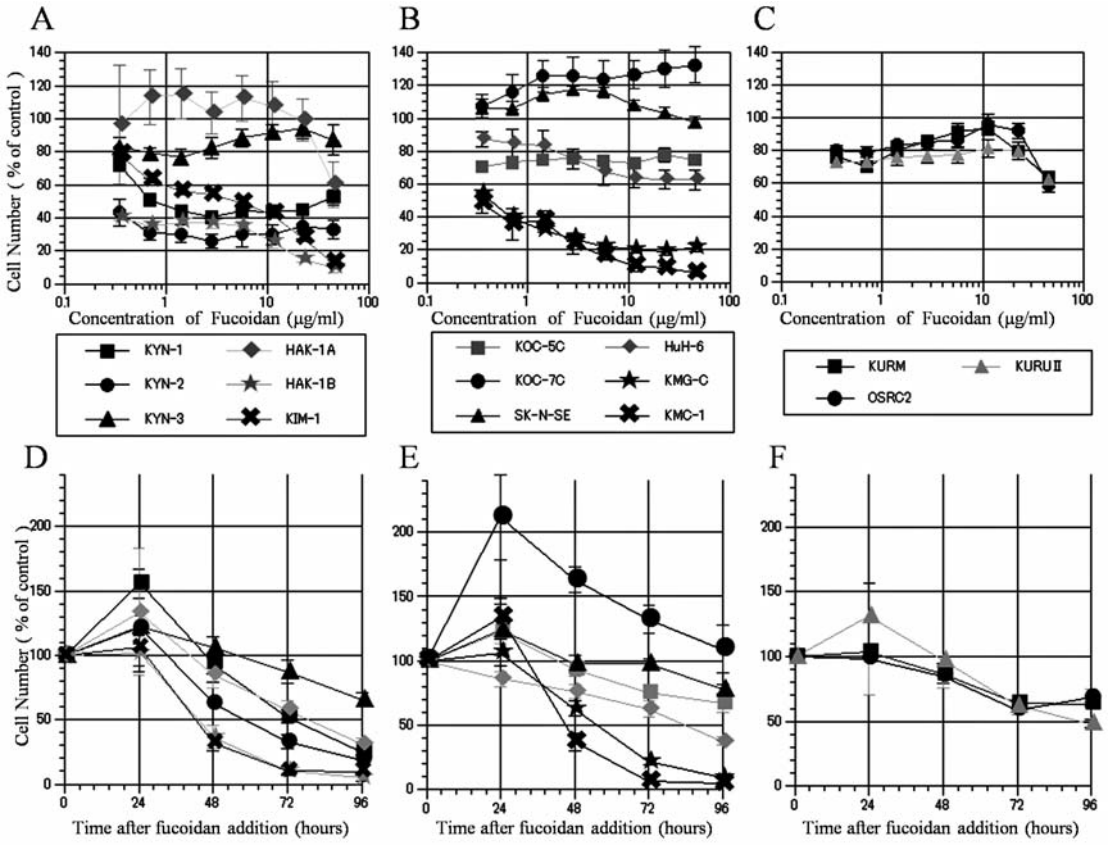

Figure 2. Antiproliferative effect of fucoidan (A-C) 72 h after 0.35, 0.70, 1.40, 2.82, 5.63, 11.25, 22.50 or 45 μg were added. Cell proliferation was suppressed in a dose-dependent manner in the 4 cell lines (KIM-1, HAK-1B, KMG-C, KMC-1), but not in the other 11 cell lines. The values represent the means ± SE of the experiments. The experiment was repeated at least twice for each cell line. (D-F) Chronological changes in the relative viable cell number (% of the control) after adding 22.5 μg of fucoidan. A time-dependent growth inhibition was observed in 13 cell lines with the exception of KOC-7C, and growth was suppressed over time to varying degrees.

in cellular viability. The IC_{50} value was calculated and used as a parameter in the comparison of the relative cytotoxicity of each cell line.

Quantitative analysis of fucoidan-induced apoptosis. Six HCC cell lines were cultured with or without fucoidan (22.5 or 90 μg/ml) for 72 h and then stained with Annexin V-EGFP (enhanced green fluorescent protein) using Apoptosis Detection Kits (Medical & Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocol. After staining, the cells were analyzed using a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA), and the percentage of Annexin V-EGFP-positive cells was determined.

Cell cycle analysis. Three HCC cell lines (HAK-1A, KYN-2, KYN-3) were cultured with or without fucoidan (22.5 μg/ml) for 24, 48 or 72 h, labeled with 10 μmol/l BrdUrd for 30 min, fixed in 70% cold ethanol at 4˚C overnight, and stained with anti-BrdUrd antibody and propidium iodide (Sigma Chemical Co., St. Louis, MO) using a previously described technique (23). The stained cells were analyzed by FACScan using the CellQuest software program (ver. 3.3, Becton Dickinson). The distribution of cells in the G_0/G_1, S, or G_2/M phase of the cell cycle was calculated and shown as a percentage of each phase.

Statistical analysis. All data were expressed as the means ± SD. For data analysis, the Student's t-test was used. A P-value <0.05 was considered to be statistically significant.

Results

Effect of fucoidan on morphological changes. Cell morphology 72 h after the addition of fucoidan solution was observed using H&E staining. The cell density in culture decreased dose-dependently (except for neuroblastoma) in order from HCC (KIM-1, HAK-1A, HAK-1B, KYN-1, KYN-2, KYN-3), cholangiocarcinoma (KMC-1), gallbladder carcinoma (KMG-C), renal cell carcinoma (KURU II, KURM, OSRC2), to ovarian cancer (KOC-5C, KOC-7C). The cell density of the neuroblastoma cell line (SK-N-SE) did not decrease at any concentration of fucoidan. As shown in Fig. 1, 3 HCC cell lines (HAK-1B, KIM-1 and KMC-1) presented dose-dependent apoptotic changes such as nuclear condensation, cell shrinkage and nuclear fragmentation.

Effects of fucoidan on cell proliferation. MTT assay revealed chronological and dose-dependent suppression of the proliferation of 4 cell lines (HAK-1B, KIM-1, KMG-C and KMC-1), chronological suppression in 9 cell lines (KYN-1, KYN-2, KYN-3, HAK-1A, KOC-5C, HuH-6, KURM, OSRC2, KURU II), and no suppression in 2 cell lines (KOC-7C, SK-N-SE) (Fig. 2).

The IC_{50} values at 72 h of culture ranged from 18.71 to 299.20 μg/ml. Levels at 72 h could not be obtained for KMG-C, KMC-1, HAK-1B and KYN-2 because >50% suppression occurred; however, at 48 h the IC_{50} was 13.33 μg for

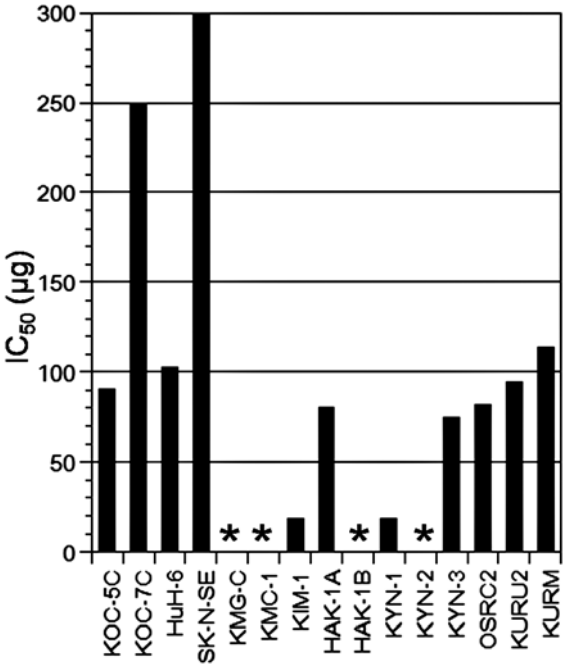

Figure 3. The IC_{50} of 15 cell lines treated with fucoidan for 72 h. *The IC_{50} values of KMG-C, KMC-1, HAK-1B and KYN-2 cell lines at 72 h could not be analyzed because the viable cell number was suppressed to <50% for all concentration levels of fucoidan.

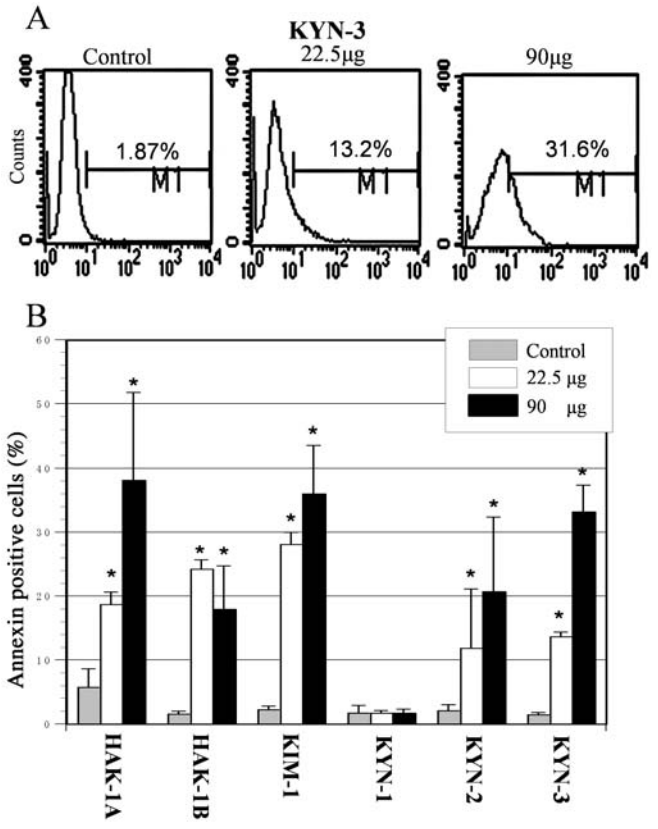

Figure 4. An analysis of apoptosis in 6 hepatocellular carcinoma cell lines treated with or without fucoidan for 72 h. HAK-1A, HAK-1B, KIM-1, KYN-1, KYN-2 and KYN-3 cell lines were treated with or without fucoidan (22.5 or 90 μg) for 72 h. The cells were harvested, labeled with Annexin-V-FITC and then analyzed by flow cytometry. (A) Percentage of apoptotic cells in the KYN-3 cell line. (B) Data represent the average (± SE) percentage of apoptotic cells. *P<0.001 vs. untreated cells.

KMC-1, 50.64 μg for HAK-1B, 52.32 μg for KYN-2 and 76.25 μg for KMG-C. According to tumor type, the IC_{50} was lowest in HCC and cholangiocarcinoma (Fig. 3).

Effect of fucoidan on apoptosis. Apoptosis was examined at 72 h of culture in the 6 cell lines that presented pronounced growth suppression in the MTT assay. The number of apoptotic cells increased significantly in the 5 HCC cell lines with the exception of KYN-1, indicating that fucoidan induced apoptosis (Fig. 4).

Effect of fucoidan on the cell cycle. Flow cytometry of the 3 HCC cell lines (HAK-1A, KYN-2, KYN-3) revealed an increased number of cells in the G_2/M phase at 72 h after the addition of the fucoidan solution (22.5 μg/ml) to the culture (Table I).

Discussion

The current study examined the anti-tumor effect of Okinawa mozuku fucoidan on 15 human cancer cell lines. Chronological and/or dose-dependent suppression of cell proliferation was observed in 13 of the 15 lines (87%). Previous studies have reported direct anti-tumor effects of fucoidan on HTLV-1-infected T cell lines and primary ATL cells (24), lymphoma cells (25) and a bronchopulmonary carcinoma cell line (NSCLC-N6) (4). The various possible mechanisms of fucoidan have also been explored, such as its anti-tumor effect induced by anti-angiogenesis (5), growth suppression due to immunopotentiation (26), and the suppression of metastasis (27,28), but only on one cell line. This current study investigated the effects had by fucoidan from the same source at the same time on multiple cell lines.

The major components of fucoidan are L-fucose and sulfate content. Previous studies used fucoidan extracted from *Fucus vesiculosus* (25), *Ascophyllum nodosum* (4,7), *Sargassum kjellmanianum* (29), *Sargassum thunbergii* (26) or *Cladosiphon okamuranus Tokida* (24), in which the percentage of L-fucose ranged from 12.6 to 36.0%, and the percentage of sulfate content from 8 to 25%. Sulfate content was also reported to be a factor with growth suppression effects (4,29). The fucoidan solution used in the current study contained these 2 substances within the above-mentioned ranges.

In this study, the suppression of cell proliferation was more apparent in the cell lines of HCC, cholangiocarcinoma and gallbladder carcinoma than in those of neuroblastoma, hepatoblastoma, ovarian carcinoma and renal carcinoma. The growth suppression effects also varied among the cell lines of the same tumor type. The IC_{50} values of the HAK-1B (HCC) and KMC-1 (cholangiocarcinoma) cell lines were additionally markedly lower in comparison to previously reported data (4,25). These findings indicate that the anti-tumor effects of fucoidan vary according to tumor type and, along with previous findings, demonstrate the anti-tumor effects of fucoidan on colon cancer but not on mammary tumors (30). This indicates that the supression of cell proliferation does not occur in all cancer cell lines. The mechanism behind the more potent growth suppression observed in the HCC and cholangiocarcinoma cell lines should therefore be explored in future studies.

Table I. Flow cytometric analysis of the effect of fucoidan (22.5 μg/ml) on the cell cycle of hepatocellular carcinoma cell lines at 24, 48 and 72 h of culture.

		24 h		48 h		72 h	
Cell line	Cell cycle	Control	Fucoidan	Control	Fucoidan	Control	Fucoidan
HAK-1A	G_0/G_1	30.7	24.3	38.4	22.8	42.9	20.5
	S	48.8	37.6	36.9	46.3	38.1	44.1
	G_2/M	16.2	22.9	18.9	22.4	13.9	19.2
KYN-2	G_0/G_1	26.1	28.8	31.9	32.2	34.0	44.4
	S	57.7	31.2	46.8	37.7	32.1	21.1
	G_2/M	12.2	28.6	9.7	20.1	18.6	23.5
	G_0/G_1	32.0	35.2	42.1	37.8	46.0	49.4
KYN-3	S	53.6	27.0	41.4	34.8	35.7	29.5
	G_2/M	9.5	31.9	11.1	23.5	12.9	17.0

Control, cells cultured without fucoidan. Fucoidan, cells cultured with fucoidan (22.5 μg/ml). Values represent the percentage of cells at each phase of the cell cycle.

Haneji *et al* (24) reported that apoptosis is induced by the activation of the caspase pathway, and Aisa *et al* (25) demonstrated anti-tumor effects accompanied by the activation of the caspase pathway and the down-regulation of the ERK pathway. In the current study, 5 of the 6 HCC cell lines presented a significant dose-dependent increase in apoptosis. The activation of caspase-3 and -9 in HAK-1B, which presented marked apoptosis, was therefore investigated. However, no clear activation was observed (data not shown), indicating that the anti-tumor effects of fucoidan on HCC cell lines could be associated with a different pathway.

With regard to cell cycle effect, Haneji *et al* (24) reported that fucoidan induced the accumulation of cells in the G_1/S phase, and Riou *et al* (4) observed that anti-tumor effects were accompanied by a G_1 phase block. On the other hand, Aisa *et al* (25) reported no effect on the cell cycle. The current findings regarding the cell cycle demonstrate for the first time an increase in cells in the G_2/M phase.

In the current study, fucoidan suppressed cell proliferation in a time- and dose-dependent manner at various degrees, and its effects were particularly pronounced in the HCC, cholangiocarcinoma and gallbladder carcinoma cell lines. The results indicate that the mechanisms of fucoidan action include the induction of apoptosis and the inhibition of the cell cycle.

At present, the clinical results of treatment for advanced HCC and cholangiocarcinoma are unsatisfactory. Fucoidan could be a potential anti-tumor remedy for specific cancers, such as HCC or cholangiocarcinoma. More detailed information on the anti-tumor effects of fucoidan should therefore be obtained in future animal studies.

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