Document on origin, discovery and usage condition in foreign country

1. Details of origin or discovery

Japanese are taking seaweed (specially brown sea weed) in their daily life from ancient period. It is very deeply familiar food in Japan. People with high intake of brown seaweed were generally thought to have a lower disease and have a long life span, although, neither the investigation nor the research were conducted on the content elements of brown seaweed too much deeply until recently. Especially, it is told that the appearance of disease rates such as cancer, autoimmunity diseases, and diabetics were extremely low who take the brown seaweed in their daily life.

USUI (1980) examined the anti-tumor effect of the hot water extracted ingredients from the brown seaweed harvested from Japan sea water by transplanting tumor cell (SR180), and reported that the proliferation of the transplanted SR180 cell is remarkably obstructed by the oral administration of the hot water extracted ingredients of brown seaweed. The elements which showed this anti-tumor effect was called Fucoidan, reported by Kylin in 1913, and the anti-tumor mechanism was guessed by the activated macrophage. Afterwards, a lot of researches on Fucoidan were performed. The research has shown the basic structure of Fucoidan which are 1→2 or united 1→3 of α-L-Fuctose-4-sulfuric acid, and the pharmacological/ biological properties were reported in various scientific topics. A basic mechanism of anti-cancer and anti-tumor effect might be the activation of base (innate) immunity and it is assumed that the anti-metastasis action is happened by the damage of newborn blood vessels as well as blocking the cell bonding factor (cell adhesion factor) such as selectin and laminin.

The mechanism of the anti-metastasis action of Fucoidan was verified by different research laboratories, however, conflicting result was reported about anti-cancer and anti-tumor effect. OKUZUMI (1991) examined the anti-cancer agents extracted from the brown seaweed and reported it as fucoxanthin which is a type of carotenoid abundantly present in the brown seaweed. Fucoxanthin obstructs the proliferation of each cell of GOTO (neuroblastoma), HGC-27(gastric cancer), HeLa(cervical cancer), and COLO 320DM (colon cancer) and SW 1116(colon cancer) remarkably and this proliferation obstruction are done by obstructing the revitalization of ornithine decarboxylase. Anti-cancer effect of fucoxanthin with the anti-cancer drug such as Torogritazon (troglitazone) were clearly documented, however, KIM (1998) clarified the chemically derived cancer prevention effect of fucoxanthin, and HOSOKAWA (1999, 2004) clarified the fucoxanthin induced apoptosis to the cancer cell where

fucoxanthin itself showed a strong anti-cancer effect.

In the year 2004, Tani from Horiuchi Ltd conducted a research on basic elements of the brown seaweed who supported the opinion of apoptosis inducement function of fucoxanthin, and he further shown the inducement of apoptosis was done by the activation of casparase. In a series of researches, they found the immunological differences of anti-cancer action between the refined and rough fucoxanthin. The changes in basic immune cells (NK, and LAK cells) and inflammatory factors (IL-1α and TNF-α) were not confirmed in refined fucoxanthin, however, a significant decreasing of inflammatory factors (IL-1αand TNF-α) and a significant increasing of activation of basic immune cells (NK, and LAK cells) were confirmed by rough fucoxanthin.

By the investigation of the content elements of fucoxanthin, it was found that the molecular weight of the elements is about 300 which combined with magnesium and tentatively called CCK300. CCK 300 clarified the situation that an immunological action of the Catsgan animal has happened because of this material which has the peculiar obstruction revitalization effects on COX-2. Moreover, regarding the tumor cell proliferation obstruction action of the elements extracted from the brown seaweed was first reported by Tani. However, a lot of researchers assumed that it depends on heparin (heparin) connectivity cellular growth factor and strong compatibility (affinity) to block cellular growth factor. Fucoidan has these special properties.

On the other hand, LOGERT (1997) reported that fucoidan stops the G0/G1 term at the cell cycle, however, DAS (2005) reported that fucoxanthin stops the G0/G1 term at the cell cycle. Tani reported that fucoxanthin has no action that can stop the cell cycle reversible and the influence on the cell cycle by the refined fucoidan was not confirmed. However, it is confirmed that the material that has caused this phenomenon is called CCK 300.

Horiuchi Ltd which had about 20 years research experiences associated with the extracted elements from brown seaweed, developed a combination product by fucoxanthin and CCK 300 in 2001, conducted the further advanced developmental research on Brown Algae Special Extract AIF (brand name and tentative name) which is stabilized by the fuoidan. Administration condition of various experiments for tumor formation and its prevention measure on the anti-tumor effect were experimented. Moreover, safety test and a physico-chemical examination of the toxicity test etc. also conducted simultaneously.

From March 2003, a joint research was performed with Horiuchi Ltd and Tanglewood Ltd in the area of production, physical properties research and standard

set experiments. Chronic toxicity test was performed in rat as there was no toxic effect was found in Brown Algae Special Extract AIF when experimented in the mouse.

In these each experiments, Brown Algae Special Extract AIF was found as a steady physico-chemical material with a wide anti-tumor spectrum which can be used on the activation of proliferation obstruction of tumor cell, an inducement of apoptosis, and the base (innate) immunity cell.

As a result of the above described experiments, the pre-clinical pilot study of Brown Algae Special Extract AIFf was conducted in the CI research society which was formed by the director of Hisashi (MUROHISA) of Hamamatsu health center and ANDO yoshiro, orthopedics department, former Kyushu National Cancer Center, in November, 2003. The minimum dosage was set as 1g/human/day based on the dosage used in the pre-clinical pilot study of Brown Algae Special Extract AIF as it is the highest safety level of sub acute toxicity showed in the experiments both on mouse and rat.

Brown Algae Special Extract AIF is the materials of 200mg fucoxanthin, 400mg CCK 300 and 400mg fucoidan as stabilizer, which are filled into the capsule. This capsule is used for the oral administration. To confirm the effect and safety, a pilot study was conducted on the progressed and terminal cancer patients during the December, 2004 to November, 2005.

Remarkable improvement of the reduction of the tumor, tumor marker's decrease, and the development of QOL etc. were confirmed by the this pilot study and reported by the CI research group in November 17, 2005. At the beginning of this pilot study, two people showed the side effects of transitory abdomen enlarged feeling (fullness feeling) and soft feces. Afterwards, this side effects were disappeared.

Feature and utility of Brown Algae Special Extract AIF

(1) Brown Algae Special Extract AIF is the materials of fucoxanthin and CCK 300 induced into the fucoidan which is extracted from brown seaweed.

(2) In case of mouse and its homogenous subjects, anti-tumor, anti-tumor cell metastasis, and life-extending effectiveness were observed by administrating the single oral dosages of Brown Algae Special Extract AIF. Moreover, it has a wide anti-tumor spectrum and has prevention effects of carcinogenesis by single administration.

(3) The mechanism of the anti-tumor effect of Brown Algae Special Extract AIF is examined in details and reported as follows:

The immediate cell toxicity to the healthy cells was not reported at all, however,

inducement of the apoptosis of the cancer cell by the route of Casparse 8 and 3 was observed.

Proliferation and the permeation of the cancer cell are obstructed by a peculiar obstruction of cyclooxygenase(COX)- 2 revitalization.

The NK cell and the LAK cell are activated, and it shows the anti-tumor effect.

It is thought that the above are the main effect appearance mechanisms of Brown Algae Special Extract AIF.

(4) Fucoxanthin and CCK 300 which composes the Brown Algae Special Extract AIF are absorbed from a digestive system. Fucoxanthin unites with retinol binding protein (RBP) in blood and CCK 300 is included into the lipo-protein and distributed in the whole body. Fucoxanthin is absorbed by the RBP receptor on the cell membrane and COX-2 is absorbed by the CCK 300 promptly into the cell.

(5) The anti-cancer action was reported in various solid cancers in the pilot study as shown in the table, and the reduction of cancer and the tumor marker in blood have also been decreased remarkably. Moreover, the improvement of QOL which recovery the appetite and relief of pain, etc.

(6) A transitory abdomen enlargement feeling is observed though it is not accepted as a main side effect.

2. Work allotment of Horiuchi Ltd and Hydrox Co. Ltd.

3. Usage condition in foreign country

Brown Algae Special Extract AIF is a medicine that discovered in Japan, however, there is no clinical usage example in the foreign country until now.

4. General name

Name of Brown Algae Special Extract AIF is derived from the combination of initial letters of Fucoxanthin and CCK 300 Complex, and Fucoidan is used in behind for indicating the meaning as stabilizer agent.

5. Comparative study of characteristic and other medicines

Brown Algae Special Extract AIF is a medicine whose principal ingredients are Fucoxanthin and CCK 300 extracted from the brown seaweed. It has anti-tumor effect and the life-extending effectiveness which are demonstrated by single administration. Moreover, it shows a plain carcinogenesis prevention effect against chemical carcinogenesis prevention measure. In addition, it is thought that the anti-tumor effect is shown by activating base (innate) immune response.

It is reported from the pre-clinical pilot study that an anti-tumor influence and life-extending effectiveness are admitted by single administration comparing with the patient who cannot be operated. Moreover, the reinforcement of life-extending

effectiveness and anti-cancer action are confirmed from the single administration as well as combining administration with various anti-cancer drug. There is no medicine existing which homogenous to the Brown Algae Special Extract AIF.

Outline B: Physical chemistry, standard, and examination method

1. Confirmation of structure

General name: Brown Algae Supecial Extract AIF

Molecular formula of fucoxanthin: $C_{42}H_{56}O_6$

Structural formula

Molecular formula and structural formula ・・・・・ under analysis.

Structural formula

Under analysis

2. Production method

The manufacturing method of Fucoxanthin is the modified method of Sandra Hanley method. That is, 99.5% ethanol is added to frozen undaria, and the final density is made 45-50 (w/w) %. Leaving it for 24 hours with stirrings. Ethanol is removed completely under a vacuum. 99.5% ethanol is added, makes it 85 %-90% in final density, and ethanol soluble material is obtained. Ethanol is removed under a vacuum, and dissolves in 30% methanol. Makes ODS column equilibrium by 30% methanol, and washes the column by 30% methanol. Each of these amount is collected as a raw material for making CCK300. In addition, after washing the column by 90% methanol, Fucoxanthin is extracted by 99.5% methanol.

CCK300 is manufactured as follows. Collected 30% methanol fractions are removed under a vacuum. The melt ability parts of ethanol (99.5%) of 45℃ are collected, crystallizes it under the environment of 4℃. Collect the crystals, washes with 99.5% ethanol and dries it.

3. Confirmation of Structure

The maximum oputical density of fucoxanthin is 449.5nm as shown in the figure below. The maximum absorption wave length of the fucoxanthin-ethanol solution is 450nm according to the Merck index (Thirteen Edition), and fucoxanthin complex is well corresponded with this range.

CCK300 is yet to be analyzed in both molecular formula and structural formula. However, it presumes that molecular weight will be about 300 even though it unites with magnesium by molar ratio 1:1.

High-speed, liquid Chromatography/Mass Spectrometry (LC MS)

Condition of Fucoxanthin

・LC;

Condition

Device: NANOSPACE SI2 (SHISEIDO)

Column: Thermo Hypersil-Keystone Hypurity C18 (5μm 150×2.1mm) Column temperature: 40℃

Movement aspect: A:80% methanol

B:100% methanol

Grageant condition: A/B 100/0 (10 mint) 0/100 (30 mint)

Flow velocity: 0.2mL/minute

Amount of injection: 5μL

Detection: 450nm

LC-MS;

Device: MSPart LCQ DECA XP plus (THERMO ELECTRON)

LC: NANOSPACE SI-2 (SHISEIDO)

Column: Thermo Hypersil-Keystone Hypurity C18 (5μm 150×2.1mm) Column temperature: 40℃

Movement : A:80% methanol

B:100% methanol

Grageant condition: A/B 100/0 (10 mint) 0/100 (30 mint)

Flow velocity : 0.2mL/minute

Injection amount: 5μL

Ionizing method: ESI

Measuring mode: Positive

Scanning range m/z 100-1500 s

Spray voltage: 4.5kV

Sheath gas: Nitrogen 75psi

Chromatography;

Condition of CCK 300

High-speed, liquid Chromatography/Mass Spectrometry (LC MS)

Condition of CCK 300

Condition

Device: NANOSPACE SI-2 (SHISEIDO)

Column: Superdex Peptide PE7.5/300 (Pharmacia)

Column temperature: 40℃
Movement aspect:　Ethanol: Water = 50:50
Flow velocity:　0.2mL/minute
Amount of injection: 50μL
Detection:　210nm
LC-MS;
Device: MS Part: LCQ　DECA　XP plus (THERMO ELECTRON)
LC: NANOSPACE SI-2 (SHISEIDO)
Column temperature: 40℃
Movement aspect:　Ethanol: Water = 50:50
Flow velocity:　0.2mL/minute
Injection amount: 5μL
Ionizing method: ESI
Measuring mode: Positive
Scanning range m/z 100-600
Spray voltage: 4.5kV
Sheath gas: Nitrogen 75psi
2. Physical chemistry etc.
・Properties
Brown Algae Special Extract AIF is a brown powder, specifically, it has no smell, however, in some cases, it has some sort of smell of seaweed with bitter taste.

Dissolubility: Brown Algae Special Extract AIF can be melted only 0.5% in hot water as it is combined with water insoluble elements called fucoxanthin.
pH: pH of the liquid is 6.6-7.2 when Brown Algae Special Extract AIF is dissolved in 0.5% water.
Moisture absorption (Figure is omitted.): Brown Algae Special Extract AIF shows some moisture absorption capability. When Brown Algae Special Extract AIF is preserved at the 100% (25℃) relative humidity condition, absorption of the moisture increased about 40% within 14 days. When Brown Algae Special Extract AIF is preserved under the condition of the relative humidity 25%, 52%, and 83%, it will be reached in equilibrium by 8%, 14%, and 21% moisture.
3. Standard and examination method
Standard and examination method of the Brown Algae Special Extract AIF are conducted by taking protection measures from mixing the impurities from raw

materials, production process and taking restriction measures for avoiding the residuals remaining. It is considered that essence should be understood as much as possible. Moreover, it is based on the examination of the measurement value and stability and follow the Japanese medicine production terms and conditions.

As the Brown Algae Special Extract AIF is the product of carotenoid (fucoxanthin) and magnesium uniting materials (CCK 300), thus the HPLC method was adopted. Regarding the fixed quantity and standard of the products, fucoxanthin is fixed by the Wako pure medicine company and CCK 300 is fixed by our company in the area of refinement and standard method. Experiment of mouse transplanting cancer was additionally set up due to confirm the effects of proliferation obstruction of apoptosis to the tumor cell, activation of NK and LAK cells and the peculiar obstruction revitalization of COX-2.

Standard, examination method of Brown Algae Special Extract AIF, set reasons, selection of six lots and the measurements values of the examinations are pin-pointed as follows:

Content;

Standard and examination method

This is a refined stabilized combined product of carotenoid and magnesium which is extracted from the seaweed. It contains 20% fucoxanthin and 40% CCK 300 at dry condition.

Set reason;

Experiment method is set up according to the HPLC method which follows the measurement regulation. As the products shows the moisture absorption ability, thus the contents of the dry products are assumed to be standard. It sets up the standard value of 96-102% considering the measurement values as well as based on the result of the standard examination.

Measurement value: Content (%) : 97.01-101.04, Mean value (%) : 98.33

σ1: 0.69

σ2 : 0.43

σ1: As for six lots, it is repeatedly mean values of the standard deviation of examination (n=3).

σ2: Standard deviation of mean value of each lot

Standard and examination method: It is brown colored powder, mostly has no smell, however, in some extent, it has some smell of sea weed and bitter taste. This product can be dissolved in 0.5% water. Reddish color carotenoid can be extracted by the

methanol, ethanol or acetone treatment. It has moisture absorption capacity.
Set reason;
Out ward appearance: brown color powder. No smell and taste, however, some cases, it has bitter taste and has slightly smell of seaweed.
Dissolubility

Liquid	Dissolubility	
Water	Dissolved at 0.5% density	
Methanol	Extracted carotenoid	Reddish brown color
Ethanol	Extracted carotenoid	Reddish brown color
Acetone	Extracted carotenoid	Reddish brown color

Moisture absorption: It absorbs the moisture
Temperature: 25±1℃
It shows 12.5-13.5%water (losses on drying) at 52% relative humidity.

Measurement
Outward appearance: Reddish brown
Smell: Very slightly smelling
Taste: Slightly bitterness
Solubility: Dissolved in water at 0.5% density
Confirmation test
Standard and examination method
99.5% ethanol of 10ml is added with 1g sample product, often stirs by the vortex, and a visible optical spectrum is measured. Maximum absorption is shown in the wave length of 445-455nm.
Set reason;
This is a combined product of fucoxanthin, CCK 300 (mg induced) and fucoidan. Confirmation, standard and examination method are set up according to the color reaction of CCK 300 with magnesium and using the maximum absorption wave length of fucoxanthin as fucoidan is used as a stabilization agent of fucoxanthin and CCK 300.
Measurement value:

Ethanol liquid	Shows red color
Magnesium reaction	Shows purple color

pH
Standard and examination method
pH of the liquid is 6.6-7.2 when 1gm of sample product is dissolved with 100ml water.

Set reason;
Measuring values can easily be changed because this combined materials are hardly shown the buffer capacity. pH 6.6-72 at neutral region was set up as a standard value after consideration of the measurement value and the difference of measurements in the stabilization test.
Measurement value: pH 6.67-7.11

Purity test and solution properties
Standard and test method:
Liquid of defined pH is suctioned by the 1nm membrane filter and no residual materials are existed in the membrane filter.
Set up reason;
Dissolubility judgment is difficult as it is liquid and brown color and that is why, solution properties were restricted by the filtration.
Measuring value: No residual materials are existed on the membrane filter.
Purity test and heavy metal;
Take 1.0g of the material, examine it according to the third law. 2.0ml lead standard product is added into the comparing liquid (20ppm or less). Set up the standard and test method for measuring the heavy metal quantity into the product. The third method of the heavy metal examination method of the Japanese general medicine examination method was adopted. The standard value was assumed to be 20ppm or less in considering with the measurement value and proper sample quantity for conducting the examination.
Measuring value: Less than 2ppm
Purity test/ Arsenic
Take 0.5g sample product and examines it (2ppm or less) according to the operating third method.
Set reason;
Standard and the examination method were set up to restrict the amount of arsenic included in the product. The examination method adopted the third method of the arsenic examination method of the Japanese general medicine examination method. The standard value was assumed to be 4ppm or less, considering the measurement value and the proper quantity of the sample for conducting examination and moreover, it follow the each article of Japanese medicine bureau.
Measuring value: Less than 2ppm

Purity test/Nitrogen

Standard and examination method

Take 0.5g precisely measured sample product, the amount of nitrogen should be 3.10% or less when examines it by the nitrogen fixed quantity method.

Set reason;

The nitrogenous substance that content in the raw material might remain because sample is a natural product. To restrict the nitrogen amount, standard and the examination method were set up. Nitrogen fixed quantity method from the Japanese medicine examination method was adopted as examination method. The standard was assumed to be 3.10% or less based on the measurement value.

Measurement value: 0.63-2.06%

Losses on drying

Standard and examination method

Standard: 7.0% or less

Set reason;

Standard and the examination method were set based on the Japanese medicine general examination method. Test condition such as, dry temperature and time are examined beforehand. The condition of 90℃, low pressure, 5-oxidation phosphorus and 5 hours time were set up. The standard value was assumed to be 7.0% or less based on the measurement value.

Measuring value: 3.25-5.41%

Anti-genic test

Standard and examination method

0.10g sample product is melted in 100ml distilled water for the injection.

5.0g glucose is added to this liquid, dissolves and sterilizes it, makes it to the sample solution. Four normal guinea pigs of 250-400g in weight are used.

Sample solution (1.0 ml) is injected in celiac of guinea pig on the 1st day, 3rd day and the 5th day. For the contrast, horse serum 0.10ml is separately injected in the celiac of guinea pigs. 2 on the 15th day, and remainder 2 on the 22nd day, 1.0ml sample solution is injected to the already sample solution injected guinea pig in their vein. 0.20ml horse serum is injected to the already horse serum injected guinea pig in their vein. Breathing difficulties, lethargy or fatal are observed after injection (30minutes/24hours after). However, sample solution injected guinea pig does not show any such situation.

Set reason;

It is a natural material. Standard concerning the antigen was set up. Administration

dosage is set up more than 25mg/1kg body weight of the guinea pig.

Measuring value: Both are negative

Anti-tumor examination

Standard and examination method

・Test animal

(2LL) cell (1×10^5) that has metastasis effect on lungs are transplanted to BALB/c57black mouse. Ten is made one group.

Sample solution

0.65mg of sample product is dissolved in the 100ml water, and sterilizes it.

Operation and judgment

After 2LL cell transplantation, 1.0ml sample solution is injected to each test animal continuously for once a day for five days. In addition, it is injected continuously for five days since seventh day after beginning the administration. Four weeks passed after having transplanted 2LL cell, it slaughters and removes the transplant part tumor and lungs. Weight it and count the lung of metastasis nests. Consider the average tumor weight (Wc) and number of average metastasis nests (Mc) of contrast group. Consider the average tumor weight (Wt) and number of average metastasis nests of sample solution administering groups (Mt). Both tumor obstruction rates and the metastasis obstruction rates will be 80% or more when each status is calculated by the following expression.

Antiproliferative effect of fucoxanthin on cultured cells

Diverse carotenoids with different chemical structure are present in edible plants in significant amounts. Fucoxanthin is one of the majoe xanthophylls in the brown algae. It have the characteristic structures of 5, 6-monoepoxide and allenic bond. Fucoxanthin has been reported to strongly inhibit the expression of N-*myc* oncogene, cell cycle progression in the human neuroblastoma cell line, GOTO cells, N-ethyl-N'-nitro-N-nitrosoguanidine-induced mouse duodenal carcinogenesis, and the growth of human promyelocytic leukemia HL-60 cell by apoptosis induction. Therefore, fucoxanthin remarkably reduces the viability of human prostate cancer cell lines, PC-3, DU 145, and LNCaP.

In this present study, we evaluated the effects of fucoxanthin on the viability of several cell lines as follows: mouse melanoma (B16), human colorectal (Caco-2), human colectal adenocarcinoma (HCT116) as cancer cells, and human normal embryonic lung fibroblast (MRC-5) and human male umbilical cord fibroblast (HUC-Fm) as normal cells.

As previously reported, fucoxanthin (All-*trans* isomer) was prepared from brown algae *Undaria pinnatifida*. MRC-5, HUC-Fm and B16 were obtained from Riken Gene Bank (Tsukuba, Japan). PC-3 abd Caco-2 were obtained from the American Type Culture Collection (Rockville, USA). HCT116 was obtained from Dainippon Pharmaceutical Co. (Osaka, Japan).

In order to evaluate the effect of the fucoxanthin on the viability of these cells, the cells were seeded at a density of 5×10^3 cells per well containing 100uL of culture medium in 96-well plates for 24h and the medium was then changed to fresh medium supplemented with the fucoxanthin. Fucoxanthin was dissolved in distilled tetrahydrofuran(THF) was added to the culture medium at a final concentration of 5 or 10uL with the final concentration of THF in the medium being 0.5%(v/v). The control culture recieved only THF (vehicle alone). After 72h of cultivation, cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Data represent the mean$\pm$standard deviation values. All experiments were done under dim yellow light in order to minimize isomerization and degradation of fucoxanthin by light erradiation.

5uM fucoxanthin significantly reduced the viability of HCT116 and PC-3 cells (table). In particular, 5uM fucoxanthin reduced the viability of HCT116 cells to 35.0% more intensively than that of PC-3 cells ($P<0.01$). At a concentration of 10uM, fucoxanthin significantly reduced the viability of HUC-Fm and Caco-2. The viability of

HCT116 cells was remarkably reduced by 10uM fucoxanthin more strongly than those of the other cells tested except for PC-3 cells ($P < 0.01$).

The present study indicated that fucoxanthin was more effective in reducing the viability of HCT 116 human colon cancer cells as well as PC-3 human prostate cancer cells than the viability of the other cancer and normal cell lines tested.

Fucoxanthin is ingested via edible brown algae. Some part of the fucoxanthin with the metabolites produced in the digestive tract would be absorbed in the jejunum, as suggested in other studies, while the other part would reach the colon through the intestinal tract. Thereby, fucoxanthin might work directly in the colon as cancer-preventing agents against some types of colon carcinoma.

Table Effect of fucoxanthin on the viability of various cells.

	Cell viability (%)					
	MRC-5	HUC-Fm	B16	Caco-2	HCT116	PC-3
control	100	100	100	100	100	100
fucoxanthin						
5uM	81	75	85	78	35	62
10uM	60	46	62	51	24	37

Cells were seeded in a 96-well plate and cultured in a 5% CO_2 humid incubator at 37℃ for 24h, and then treated with THF alone and fucoxanthin. After 72h of culturing, cell viability was evaluated by means of a MTT assay. The controls were cultured with the vehicle alone (THF).

AIF suppresses azoxymethane-induced rat colon tumorigenesis

We investigated the modulatory effect of AIF containing fucoxanthin and CCK on colon carcinogenesis initiated with azoxymethane(AOM) in rats. Starting one week after the initiation, rats received the diet mixed with 1 or 5 mg/kg-body weight AIF for 34 weeks. The inhibition rates of colonic adenocarcinoma in rats that received 1 or 5 mg AIF after AOM exposure were 57% and 88%, respectively. Also AIF feeding supressed the prostahlandin(PG)E_2 levels and cell proliferation activity, and enhanced apoptosis in colon adenocarcinomas. These results suggested that AIF might be a possible chemopreventive agent colon cancer development.

In a recent literature review of brown algae extracts, especialy fucoxanthin, CCK and fucoidan, a broad spectrum of biological activities including anti-carcinogenesis and anti-tumor activities were discussed. Epidemiological studies have revealed that brown algae intake is correlated with a reduced risk of certain types of cancer. Fucoxanthin and CCK contained AIF has anti-tumor/cancer effect. It is known that fucoxanthin and CCK possesses certain biological activities such as induction of apoptosis, reduction of proliferative and COX-2 inhibiting activity in various cancer cells.

Colorectal cancer, a common malignancy worldwide, is an important contributor to cancer morbidity and mortality, and to overall international cancer burden. This malignancy has increasing in Asia owing to the changes in life style including dietary habit of increased meat consumption. In Japan, colon cancer is one of the most important causes of death: the mortality rates were 16.32/100,000 for men and 9.74/100,000 for women in 2000. In spite of several basic or clinical challenges to control colonic cancer death, no significant prolongation of survival has been obtained.

Although the content of fucoxanthin, CCK and fucoidan in brown algae is not abundant, these compounds might have great potential as a cancer-preventing (or anticancer) agent. In present study, we conducted an *in vivo* long-term experiment to invested the modifying effect of AIF on AOM-induced rat colon carcinogenesis. Since cell proliferation plays an essential role in carcinogenesis, measured cell proliferation activity using biomarkers such as PCNA-labeling index and polyamine level in colonic tumors and non-lesional colonic mucosa. To elucidate the mechanism of the modulating effect of AIF on colon carcinogenesis, apoptotic index was also assayed because certain chemopreventive agents exert inhibitory action via induction of apoptosis. In addition, PG-E2 levels were measured in colonic adenocarcinomas and their surrounding colonic mucosa since close association between the high level of

PG-E_2 and colonic carcinogenesis has been suggested.

RESULTS

General observation

Throughout the experiment, clinical signs of toxicity and poor condition were not observed in any rats. These observations were confirmed by histological examination of major organs (liver, kidny, heart and lung). Body and liver weights showed no significant differences among the groups. Intakes of the diets with or without AIF were not significantly different (these data not shown), suggesting that rats could tolerate the diets without experiencing adverse effects from consumption of 1 or 5 mg/kg・body weight AIF.

Incidence and multiplicity of intestinal neoplasm

The incidence and multiplicity of colonic adenocarcinoma in the AOM alone group were 67% and 1.33±1.28/rat, respectively. By administration of 5 mg/kg・body weight AIF, the incidence (22%) and multiplicity (0.17±0.28/rat) were significantly reduced compared with AOM alone group. The differences on the incidences and multiplicities of colonic adenoma among 1 mg AIF group were not statistically significant. AIF was suppressed the development of colonic adenocarcinomas induced by AOM.

Polyamine content and PGE_2 level in colonic mucosa

The data on assays of polyamine content and PGE_2 level in the colonic mucosa determined at the end of the study are summarized in table 2.

Table 2 Polyamine content and PGE_2 level in colonic mucosa

	polyamine(nmol/mg protein)	PGE_2(pg/mg protein)	
		normal	adenocarcinoma
No treatment group	13.5 ±1.0	62.0±15.5	-
AOM alone group	16.8±1.2	97.0±22.4	111.5±35.2
1mg AIF group	14.7±0.5	78.5±16.1	77.6±20.7
5mg AIF group	13.9±0.8	67.5±20.2	64.9±15.3

As for the total polyamine content of colonic mucosa wethout tumors, significant difference was found between AOM alone group (16.8±1.2) and no treatment group (13.5±1.0). Polyamine content of 1mg AIF group (14.7±0.5) and 5mg AIF group (13.9±0.8) for non-lesional colonic mucosa were significantly smaller than AOM alone group. The PGE2 content in colonic mucosa without tumor in AOM alone group (97.0±22.4) was significantly higher than no treatment group (62.0±15.5). As for the colonic adenocarcinomas, the PGE2 content of 5mg AIF group (64.9±15.3) was remarkably lower than AOM alone group (111.5±35.2).

High levels of PGE_2 stimulate colon carcinogenesis. Cell proliferation and apoptosis are also modulated by PGE2 production. The PGE2 content of colonic adenocarcinoma in AOM alone group was higher than AIF group. It is known that PGE_2 is produced from arachidonic acid by cyclooxygenase (COX). COX-2, an inducible enzyme, plays a central role in the production of PGE2 at inflammation sites, and is involved in colorectal cancer development. Our current study showed that CCK contained AIF selectively inhibit COX-2. The potential roles of COX-2 inhibitors in the chemoprevention and treatment of colorectal tumors are well discussed. CCK contained AIF might exert a similarly effect with selective COX-2 inhibitors at in vivo. Furthermore, CCK contained AIF interferes with gene expression of proinflammatory cytokines such as IL-1α and ß and TNF-α, which are known to be inducers of PGE_2.

name	sex	year	symptom
N	♀	48	colon cancer, metastasizes to large intenstine and lymph. After 5 weeks intaken AIF, these cancer were clearly reduced.
K	♂	85	lung cancer(carcinoma planocellulare and small cell carcinoma), After 5 weeks intaken AIF, these cancer were disappeared on CT. (Pro-GRP:600→43.4, NSE:18→6.1)
N	♂	59	gastric cancer, metastasizes to diaphragm. After 5 weeks intaken AIF, these cancer were disappeared on CT. (CA19-9:294→51.4, CEA: 11.7→3.7).
Y	♀	79	uterine cancer, metastasizes to liver, After 7 weeks intaken AIF, these cancer were reduced. (CA125:742→33.4).
N	♂	57	prostatic cancer, After 4 weeks intaken AIF, PSA was decreased to normal.(PSA:35.1→2.7).
S	♂	83	prostatic cancer, After 4 weeks intaken AIF, PSA was decreased to normal.(PSA:29.5→0.35).
N	♂	74	prostatic cancer, After 6 weeks intaken AIF, PSA was decreased.(PSA:47.2→7.1).
T	♀	84	bladder cancer. After 3 weeks intaken AIF, CEA was decreased(CEA:37→＜5,CA19-9→＜5).
S	♀	65	mammary cancer, After 5 weeks intaken AIF, the marker was decreased(CEA:74.8→28).
T	♀	67	lung cancer, metastasizes to bone and lymph. After 10 weeks intaken AIF, the marker was deceased (Pro-GRP: 70.3→23.4, CEA: 253→4.7).
K	♂	70	lung cancer(non small cancer), The cancer marker gradually decreased(Pro-GRP:60→43)

Anticancer effect of AIF

Our current study and other researcher reported that fucoxanthin induced an apoptosis for the several cancer cells. it is also known that fucoxanthin suppresses the ornithine decarboxylase which is rate-limiting enzyme of polyamine synthesis. CCK showed COX-2 selective inhibiting activity, and the synthesis of PGE_2 is inhibited *in vivo*. By combining fucoxanthin and CCK, the anticancer drug was made, named AIF. The test in the mouse which transplanted *Lewis Lung Cancer* (2LL) was carried out in order to examine the therapy effect of AIF.

Constituents of AIF which is the subject material are as follow (table 1).

Table 1. Constituents of AIF

	Concentration(%, w/w)
fucoidan	55.6
CCK	20.0
fucoxanthin	10.0
ash	14.4

Animal experiment

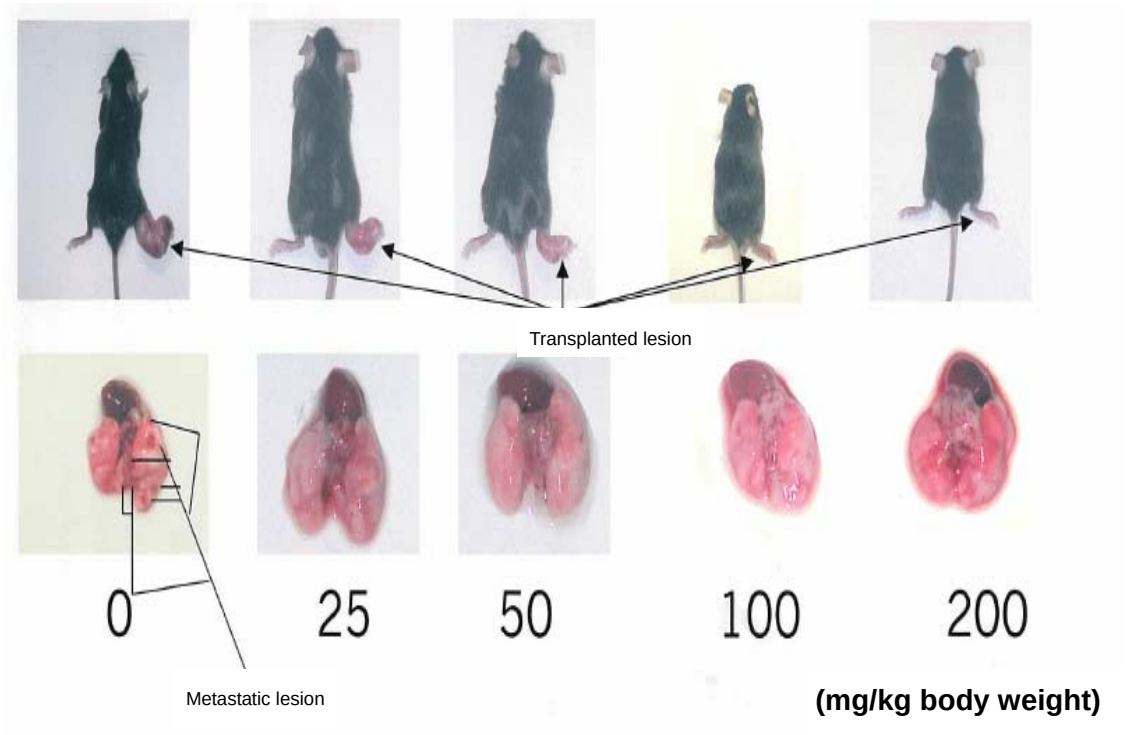
Mouse (C57BL/6、male、4week-old) was orlary administrated the diet mixed with 0 to 200mg of AIF/kg body weight feeding everyday. After 1 week, 2LL cells were transplanted in foot-pad of mouse. The heparin added venous plasma was got on 2LL cell transplantation post-third week, and it was made to be a sample of the flow cytometory.

Total T-cell, helper T-cell, suppressor T-cell, NK cell, LAK cell and B-cell were analyzed with FACS Calibur.

RESULTS AND DISCUSSION

The overall observation of whole-body and lung in 3 weeks was shown in Fig.1.

Fig.1 The macroscopic observation of transplanted post-third week



While the size of the tumor of non-administration AIF group was 12× 7 mm, it of administrated AIF was reduced with8×4mm (25mg AIF), 6×3mm(50mg AIF), 5× 2mm(100mg AIF) and trace (200mg AIF), respectively. There was the dose response at the concentration of AIF administrated with the size of the tumor. Especially, in the 50mg AIF group, the reduction of the tumor was remarkable, and the tumor cell could not seldom recognized at over 100mg AIF administration in tumor(edema). These results were summarized in Fig.2.

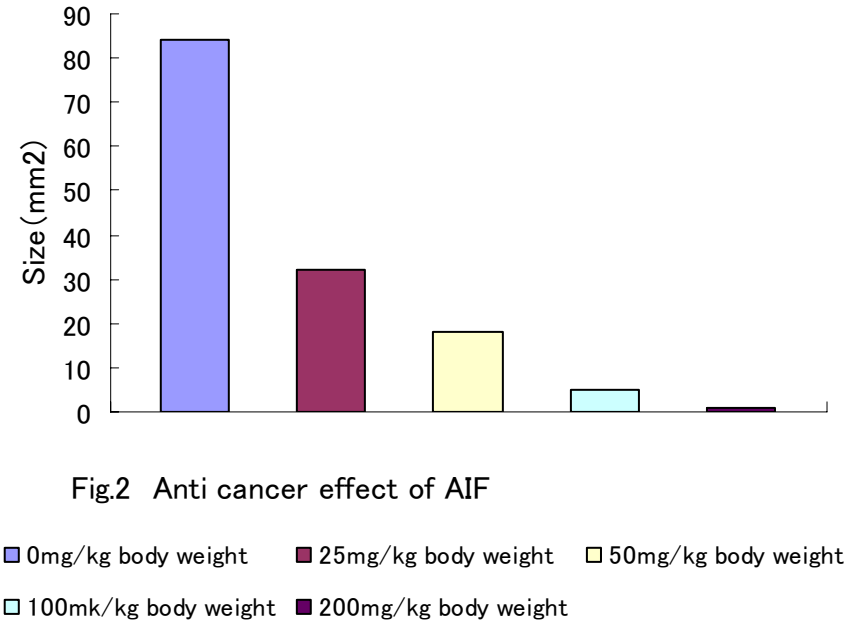


Fig.2 Anti cancer effect of AIF

On the metastasis to the lung, though in AIF non-administration group, over of 8 was observed the metastatic lesion, in AIF administrated group, the metastatic lesion could not be observed in 25mg AIF group. From these results, AIF was regarded as not only that it shows the remarkable anticancer effect but also that it strongly has the anti-metastatic action.

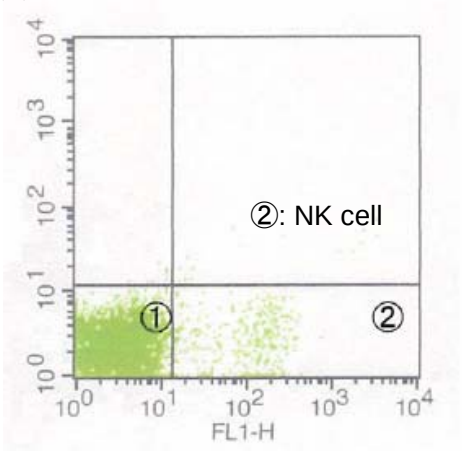
The proportion of T-cell of the AIF non-administration group was 9.8%. For this, in the AIF administrated group, it was 17.0% in 200mg AIF, and this phenomenon showed the density dependence. While the ratio of helper T-cell and suppressor T-cell was 0.83 in the AIF non-administration group, on it of the AIF administrated group, 0.84(25mg AIF), 1.06(50mg AIF), 1.24(100mg AIF) and 1.27(200mg AIF), respectively. AIF might improve the helper T-cell/suppressor T-cell ratio, because it of the normal mouse was 1.26.

Table 2 The ratio of the immunity cell

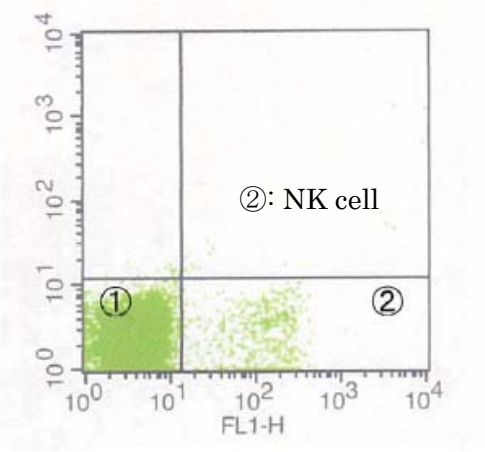
	0mg/kg body weight	25mg/kg body weight	50mg/kg body weight	100mg/kg body weight	200mg/kg body weight
T cell	9.8	10.3	14.6	15.9	17.0
Helper T cell	3.4	6.9	5.7	15.3	10.2
Suppressor T cell	4.1	8.2	5.4	12.3	8.0
NK cell	2.4	2.8	5.8	6.9	7.7
LAK cell	15.9	19.9	30.0	46.7	47.3
B cell	41.8	39.9	38.2	18.5	11.9

The analytical result of flow cytometory of NK and LAK cell was shown in Fig.3.

(1) NK cell

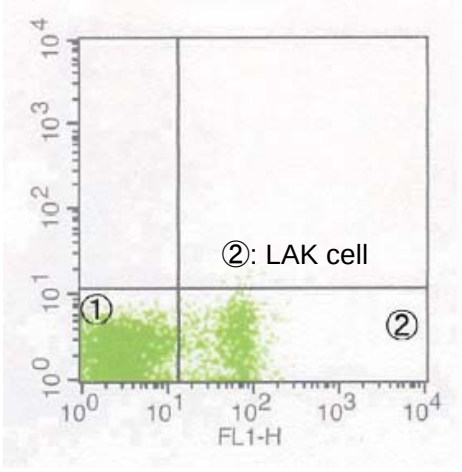


Without AIF

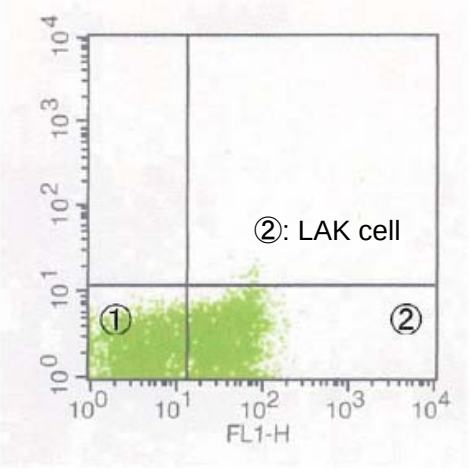


200mg AIF

(2) LAK cell



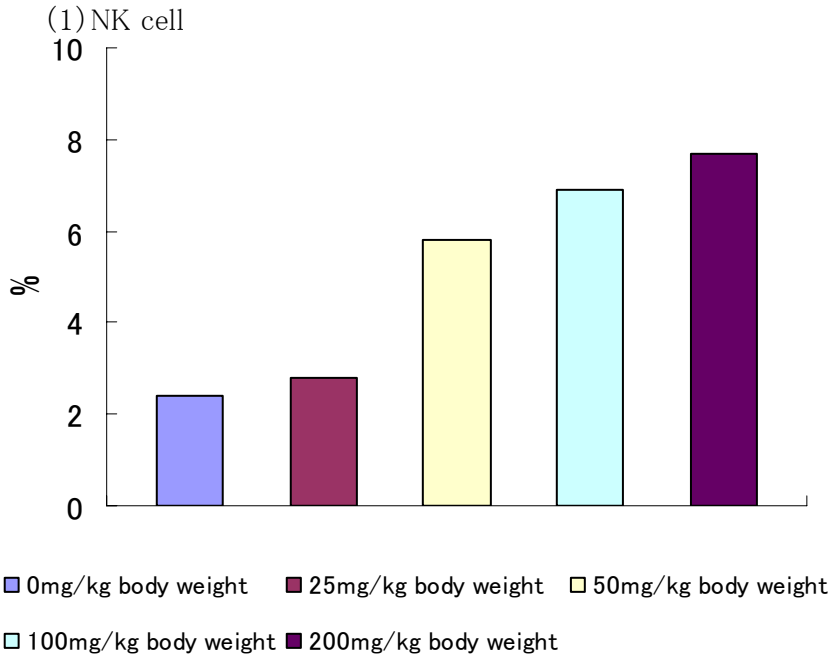
Without AIF



200mg AIF

Fig.3. NK and LAK cell analysis with Flow cytometory

Both NK and LAK cell were increased by AIF administration. This proportion of cell was summarized in Fig.4.



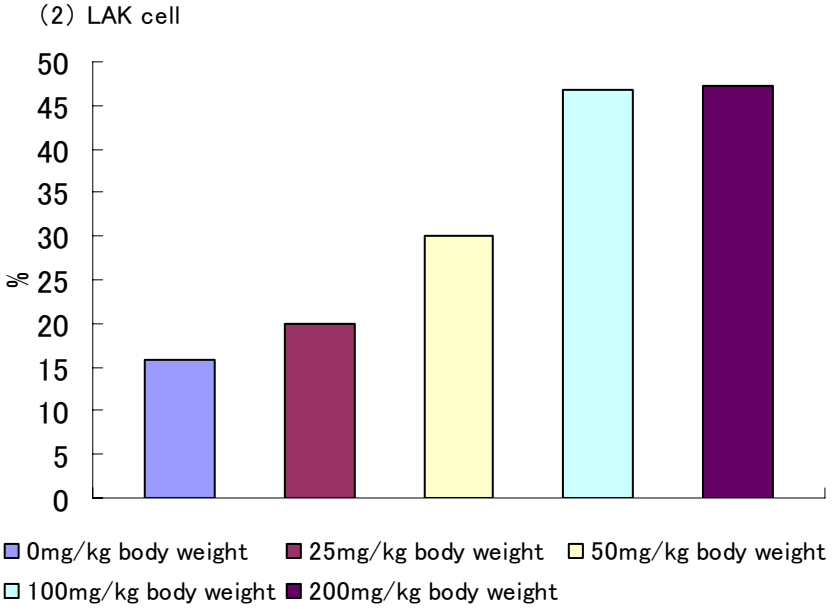


Fig. 4 The rate of NK cell and LAK cell in third weeks

NK cell kills cancer cell by the cytotoxicity. While the proportion of NK cell of the AIF non-administration group was 2.4, in the AIF administrated group, the proportion of NK cell was much higher than the AIF non-administration group. This phenomenon seems to originate for CCK including AIF.

LAK cell is also killer cell induced at IL-2, the cytotoxicity is non-specially shown for

various cancer cells. In the AIF administrated group, it was 47.3%, through it was 19.9% in the AIF non-administration group, and there was a remarkable difference.

From these facts, the strongly anticancer activity of AIF seems to have appeared by synergistic effect of fucoxanthin and CCK.