Effect of Fucoidan on the Biotinidase Kinetics in Human Hepatocellular Carcinoma

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Abstract. *Background: Hepatocellular carcinoma (HCC) is difficult to treat with anticancer drugs. Therefore, development of new drugs for HCC is required. Materials and Methods: The livers of 14 hepatoma patients accompanied by hepatitis B (2 cases) and hepatitis C (12 cases) were used. The biotinidase kinetics of HCC tissues were compared to those of the adjacent liver tissues of 13 liver cirrhosis (LC) and 1 chronic active hepatitis (CAH). Results: The Kip (the inhibition constant by biotin) of HCC tissues were consistently higher than those of LC (plus CAH) tissues: the Kip was 450±231 μmol/l in HCC tissues and 240±111 μmol/l in LC (plus CAH) tissues, p<0.001. This increase of Kip is considered to be due to an increase of biotin repulsion by biotinidase in the HCC tissues. Fucoidan, a sulfated poly-fucose, was found to decrease the Kip of biotinidase in HCC tissues, and conversely to increase it in LC tissues. Fucoidan was also found to decrease the Kip of the hepatoma HuH-6 cells. Conclusion: These findings suggest that fucoidan has a potential therapeutic effect on HCC.*

Biotinidase (EC 3.5.1.12) (1, 2), ubiquitous in mammalian cells (3, 4), hydrolyzes biocytin to biotin and lysine. Human serum and urine biotinidase is a glycoprotein enzyme (1, 5), and sialic acid of the glyco-chain increases the affinity to the biotin-amide substrate in human serum biotinidase (unpublished observation). Previously, it was reported that serum biotinidase activity significantly decreased in patients with liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (6); however, its significance in the pathogenesis of LC and HCC have not yet been determined. Since the biotin concentration is higher in cancerous tumors than that of normal tissue (7, 8), biotinidase should become resistant to the increased product (biotin) concentration in order to perform better substrate handling. A new biotinidase assay method has recently been developed using high-performance liquid chromatography (4), and this method has been applied to determine the biotinidase kinetics in HCC patients in this text.

Fucoidan, a sulfated poly-fucose, has become a matter of great interest for cancer therapy. The mechanisms by which fucoidan exhibits anticancer activity are related to its ability to suppress the proliferation of cancer cells, modulate the immune responses, inhibit tumor angiogenesis and to induce the apoptosis of tumor cells (9-15). More recently, it was reported that fucoidan exhibits antitumor activity toward Huh 7 hepatoma cells through down-regulation of CXCR12 expression (16).

In addition, Mekabu fucoidan (me-Fucoidan) had a higher binding affinity for basic proteins such as histone H2B, and inhibited A-kinase-mediated phophorylation *in vitro* (17). Fucoidan is a negatively charged polysaccharide, and biotinidase has a negatively charged sialic acid in the glycol chains. Therefore, the effects of fucoidan on the biotinidase kinetics in liver samples obtained from HCC patients and in hepatoma cell lines were also investigated.

Materials and Methods

Chemicals and reagents. Biotinyl-6-aminoquinoline (BAQ), bovine serum albumin (BSA), carrageenan lota (type V; from *Eucheuma spinosa*) and diethylnitrosamine (DEN) were purchased from Sigma (St. Louis, MO, USA). 6-Aminoquinoline was from Aldrich (Milwaukee, WI, USA). Polyoxyethylene cetyl ether (Brij 58) was from Nacalai Tesque (Kyoto, Japan). Chondroitin sulfate C sodium salt, dextran (Mr 200000~300000), heparin (from porcine small

Dedication: In memory of the usual encouragement of my beloved daughter Reiko Hayakawa (21 November 1979 – 1 February 2007).

Abbreviations: HCC, hepatocellular carcinoma; LC, liver cirrhosis; *K*ip, inhibition constant by product of biotin; *A*mo, affinity for substrate; *R*ep, repulsion to product; *C*ap, enzymatic capacity; HPAC, high-performance affinity chromatography; DEN, diethylnitrosamine; BAQ, biotinyl-6-aminoquinoline.

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intestine), hyaluronic acid sodium salt (from rooster comb), L(–)-fucose, and D-biotin were from Wako (Osaka, Japan). Fetal bovine serum (FBS) was from Moregate BioTech (Bulimba, Australia). Fucoidan was prepared from *Cladosiphon okamuranus* as described previously (18). Purified fucoidan contained less than 0.1% of contaminated proteins as determined by the microsequencing method (19).

Liver samples. HCC samples and corresponding non-tumorous tissues were obtained from 14 patients (12 male and 2 female) ranging in age from 49 to 66 years during surgery (9 samples) or autopsy (5 samples) at Gunma University Hospital and Nishi-Gunma Hospital. The liver samples were immediately stored at –80˚C. The grade of differentiation of HCC was determined pathologically. Patient data are summarized in Table I. Liver cirrhosis was found in adjacent liver tissues except for one patient (No.10, chronic active hepatitis, CAH). Normal liver samples were obtained from four patients with metastatic liver tumor and one hepatolithiasis patient during surgery (Controls 3, 5) or autopsy (Controls 1, 2, 4). Non-cancerous liver tissues demonstrating normal histological findings served as a control (Table I). Informed consent was obtained from all patients and/or their relatives. This study was approved from the institutional committee for the study of human rights.

Cell culture. HuH-6 cells (hepatoma from a 1-year-old Japanese male) were purchased from Rikagaku Kenkyusho (Tsukuba, Ibaragi, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin G (100 U/ml) and streptomycin (100 U/ml) in a humidified 37˚C/5% CO_2 incubator. The enzyme kinetics of biotinidase were analyzed using cell homogenates containing 59 μg/ml of protein in the final reaction buffer.

Protein concentration. Protein concentrations of the human livers and cultured cells were all determined by SEC protein determination method (20).

Enzyme kinetic analysis. Liver homogenate and cultured cell homogenate were prepared as described previously (5, 19, 20). Supernatant and membrane (nuclear membrane) fractions were prepared by ultracentrifuging the homogenate at 100000 xg for 90 min at 4˚C. Biotinidase kinetic analysis was performed as described by high-performance liquid chromatography (HPLC) with fluorometric detection (5). The molecular mass and number of active centers of biotinidase were determined to be 66,000 and ones, respectively. *A*mo (*k*cat/*K*m), *K*m, *K*ip, *R*ep (*k*cat x *K*ip x 10^{-3}), and *C*ap ((*A*mo x *R*ep)$^{1/2}$) were defined as follows, *A*mo is the affinity to the biotinyl substrate, *K*m is also the affinity to the biotinyl substrate (BAQ), *K*ip is the competitive inhibition constant by biotin or product inhibition, *R*ep is the repulsion power against the biotin product and *C*ap is the total capacity of the enzyme to handle the biotinyl substrate per second per enzyme molecule, respectively (5).

Fucoidan test. A fucoidan test was carried out using 2 HCC tissue samples (No. 11, 12), 3 LC samples (No. 11, 12, 13) and 2 normal livers (Controls 2, 5). Fucoidan (100 or 200 μg/ml) was added to fractions and homogenates of liver samples. To compare the effect on biotinidase kinetics, other polysaccharides such as chondroitin sulfate, hyaluronic acid, carrageenan, dextran, heparin and L(–)-fucose were evaluated.

Table I. *Characteristics of HCC and normal livers used in this study.*

Liver samples	Virus	Age (years)	Gender	Degree of differentiation of HCC
HCC				
1	+HBV	59	Male	Well-differentiated
2	+HBV	53	Male	Well-differentiated
3	+HCV	65	Male	Moderately differentiated
4	+HCV	58	Male	Moderately differentiated
5	+HCV	49	Male	Moderately differentiated
6	+HCV	61	Male	Poorly differentiated
7	+HCV	58	Male	Moderately differentiated
8	+HCV	51	Male	Moderately differentiated
9	+HCV	64	Male	Moderately differentiated
10	+HCV	63	Female	Moderately differentiated
11	+HCV	59	Male	Moderate differentiated
12	+HCV	55	Male	Poorly differentiated
13	+HCV	66	Female	Well-differentiated
14	+HCV	66	Male	Well-differentiated
Normal liver (control)				Tissue source
1		62	Male	liver metastatis from stomach cancer
2		72	Male	stomach cancer
3		43	Female	liver metastatis from gut cartinoid
4		67	Male	liver metastatis from colon cancer
5		71	Female	hepatolithiasis

In addition, HuH-6 cells were subjected to the fucoidan test. Fucoidan was added to the culture medium at a concentration of 200 μg/ml, and the biotinidase kinetics were calculated.

Determination of biotin in diethylnitrosamine (DEN)-induced HCC in LEW rat. Male LEW rats (weighing 200 g; 7 weeks of age) were purchased from Sankyo Labo Service Co., Tokyo, Japan. They were kept in the animal facility of National Institute for Child Health and Development, with a 12-h light/dark regime, and were maintained on a standard CE-2 feed purchased from Japan Clea (Tokyo, Japan).

Rats were randomized into 2 experimental groups of 3 animals each. Group 1, which served as the control, had access to regular drinking water *ad libitum*. The control group received no diethylnitrosamine (DEN). Group 2 was injected intraperitoneally with DEN at 50 mg/kg (body weight) once a week for 16 weeks by the procedure of Schiffer *et al*. (21). Two weeks after the last injection (to allow recovery from acute necrosis), all rats were anesthetized with ether. The tumor lesion and the adjacent liver tissues were surgically removed and weighed. A random piece of liver from each lobe was fixed in 10% buffered formalin and embedded in a paraffin for histological analysis. In addition, the liver was frozen immediately in liquid nitrogen for the analysis of biotin and biotinidase. Amounts of total and free biotin in the rat livers were determined by a newly developed high-performance affinity chromatographic (HPAC) method (22).

All the protocols were carried out in accordance with ethical guidelines for laboratory animals of the National Research Institute for Child Health and Development.

Table II. *Typical biotinidase kinetics of human livers.*

Specimen	*A*mo (l/mol × 1/s)	*K*m (μmol/l)	*V* (μU/mg)	*K*ip (μmol/l)	*R*ep (mol/l × 1/s × 10^{-3})	*C*ap (1/s)
Normal liver	4.4±1.5	15.4±11.0	46.6±24.0	364±107	16.2±4.3	8.4±1.3
Liver cirrhosis	7.6±5.8	9.8±5.7	47.5±30.3	240±111	14.9±11.4	10.0±6.7
Hepatoma	9.9±9.3	9.4±7.2	57.2±42.9	450±231#	29.1±28.3	15.7±14.0

Homogenates were used in these experiments. #$p<0.05$ Compared with liver cirrhosis.

Statistics. Non-parametric statistical analysis was performed. The Wilcoxon test was used to compare the kinetic values of LC and HCC samples. Mann-Whitney's *U*-test was also used to compare differences between two groups. For all tests, $p<0.05$ was regarded as significant.

Results

Biotinidase kinetics of human livers (Table II). All HCC patients' livers and 4 normal livers (except Control 5) showed *K*ip values of biotin, and *R*ep and *C*ap were then calculated. The mean *K*ip values were highest in HCC tissues, followed by normal livers and LC tissues. The *K*ip value was consistently higher in the HCC tissue than in the LC (plus CAH) tissue in each patient (Figure 1). The mean values of *K*ip were significantly ($p<0.01$) higher in the HCC tissues than in LC (plus CAH) tissues. The *K*ip values were not significantly different between normal liver and LC and HCC tissues. *R*ep and *C*ap were significantly ($p<0.05$) higher in HCC tissues than LC (plus CAH) tissues. However, *A*mo, *K*m and *V* did not differ among normal liver, LC (plus CAH) and HCC tissues.

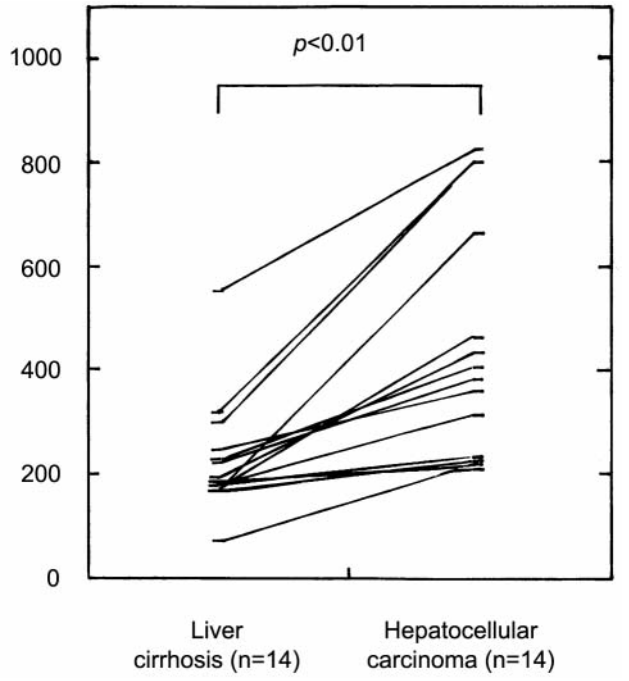

Figure 1. *Typical differences in the Kip values between LC and HCC tissues of each patient's liver. The longitudinal axis shows Kip values (μM). The Kip value is consistently higher in the HCC tissue than in the LC (plus CAH) tissue in each patient.*

Effect of several polysaccharides on biotinidase kinetics in HCC tissue (Table III). In the supernatant fraction of two HCC tissues, the *K*ip, *R*ep and *C*ap values were markedly reduced by fucoidan. Heparin also reduced the *K*ip, *R*ep and *C*ap values, while L-fucose, chondroitin sulfate, hyaluronic acid and carrageenan did not alter these values.

Effect of fucoidan on biotinidase kinetics in HCC tissue. The effect of fucoidan on biotinidase kinetics in the supernatant of one HCC tissue is shown in Table IV. Although a slight increase of *A*mo and a decrease of *K*m was observed, the capacity of biotinidase (*K*ip, *R*ep and *C*ap) was significantly reduced by fucoidan ($p<0.05$).

Effect of fucoidan on biotinidase kinetics in LC tissues. The effect of fucoidan on the biotinidase kinetics in the supernatant of three LC tissues was studied (Table V). Fucoidan activated LC biotinidase, *i.e.* although a decrease of *A*mo was observed, biotinidase capacities (*K*ip, *R*ep and *C*ap) all significantly increased in the LC tissues ($p<0.05$).

Effect of fucoidan on biotinidase kinetics in normal liver. The effect of fucoidan on the biotinidase kinetics of two normal livers was tested (Table VI). Although not sufficient for statistical analysis, fucoidan did not seem to affect the normal livers.

Effect of fucoidan on biotinidase kinetics in HuH-6 cells. The effect of fucoidan on HuH-6 hepatoma cells was tested, and the results are summarized in Table VII. Fucoidan in DMEM reduced the biotinidase capacities relating to the repulsion of biotin product (*K*ip, *R*ep and *C*ap).

Table III. *Effect of several polysaccharides on biotinidase kinetics in HCC tissues.*

Polysaccharides	*A*mo	*K*m	*V*	*K*ip	*R*ep	*C*ap
Experiment 1: Homogenate of HCC of No. 11						
Without addition	3.9	5.1	16	472	9.4	6.1
Fucoidan	4.9	4.7	18	256	5.9	5.4
L-Fucose	3.8	5.9	18	444	9.8	6.1
Experiment 2: Supernatant fraction of HCC of No. 12						
Without addition	42.2	6.8	225	452	129	73.8
Fucoidan	57.5	4.8	217	255	70.1	63.5
Chondroitin sulfate C	40.3	7.1	227	405	117	68.5
Experiment 3: Supernatant fraction of HCC of No. 12						
Without addition	42.4	6.5	217	302	83.1	59.4
Fucoidan	40.6	6.4	204	264	68.3	52.6
Hyaluronic acid	36.4	6.8	194	353	86.7	56.2
Experiment 4: Supernatant fraction of HCC of No. 12						
Without addition	37.2	7.1	208	416	110	63.9
Fucoidan	44.5	7.1	250	205	65.0	53.8
Carrageenan	35.8	8.9	250	416	132	68.7
Dextran	35.3	7.5	208	426	112	62.9
Experiment 5: Supernatant fraction of HCC of No. 12						
Without addition	43.5	6.9	238	360	109	68.8
Heparin	63.7	3.8	192	268	65.2	64.5

Units are the same as in Table II. Fucoidan, L(–)-fucose, chondroitin sulfate C, hyaluronic acid, carrageenan, dextran, and heparin were used at final concentrations of 100, 1000, 200, 200, 200, 200, and 200 μg/ml, respectively.

Increased free biotin (product of biotinidase) in the HCC tissue of rat. Using LEW rat, HCC was induced by diethylnitrosamine (DEN) and the adjacent liver tissues diffusely showed degenerative nodules in accordance with LC (21). Biotin was determined by a newly developed HPAC method (22). The results are summarized in Table VIII. Free biotin significantly increased by about 10-fold in the HCC tissue as compared to the normal rat liver.

Discussion

The present study clearly demonstrated that biotinidase affinity to the substrate (*A*mo, *K*m) and specific activity (enzyme amount; *V*) were unchanged in the HCC tissues but that the product repulsion power (*K*ip and *R*ep) and enzyme capacity (*C*ap) increased. It is of interest that the *K*ip value was consistently higher in the HCC tissue than in the LC (plus CAH) tissue in each patient (Figure 1). If the biotin concentration is higher in HCC (7, 8), biotinidase should become resistant to the increased product (biotin) concentration in order to perform better substrate handling. As shown in Table VIII, a 10-fold increase of free biotin (biotinidase product) in the HCC tissue in the DEN-treated rats was demonstrated. Furthermore, the increase of biotin in the medium of HeLa cells was reported previously by Keränen (23), and the increase of biotin in the DMEM medium using cultured liver cells was confirmed (data not shown). Thus, it is considered that biotinidase capacities in the HCC tissues must change to enhance handling of biotin (product: increased *K*ip and *R*ep) and the biotinyl substrate (substrate: increased *C*ap).

Table IV. *Effect of fucoidan on the supernatant of HCC tissue of No. 12.*

Experiment	*A*mo#	*K*m#	*V*	*K*ip#	*R*ep#	*C*ap#
1 Control	38.3	8.3	250	385	122	68.3
+ Fucoidan	44.3	6.0	208	287	75.6	57.9
2 Control	42.2	6.3	208	371	97.9	64.3
+Fucoidan	45.9	5.5	198	242	60.6	52.8
3 Control	43.3	5.4	185	377	88.3	61.8
+Fucoidan	49.2	5.2	200	239	60.6	54.6
4 Control	39.7	6.8	213	342	92.3	60.3
+Fucoidan	43.2	4.5	179	259	49.9	46.4
5 Control	39.4	6.9	213	497	134	72.7
+Fucoidan	41.5	6.6	217	442	121	71.0
6 Control	41.8	7.6	250	382	121	71.1
+Fucoidan	53.2	5.0	208	252	66.4	59.4

Units are the same as in Table II. Fucoidan was used at a final concentration of 200 μg/ml. Using fucoidan (n=6), # is significantly different from the control ($p<0.05$) by Wilcoxon test.

Fucoidan has been reported to exhibit antitumor and anti-metastatic activities *in vivo* and *in vitro* (9-17); therefore, whether fucoidan and other polysaccharide reagents

Table V. *Effect of fucoidan on the supernatant of LC portion of No. 11, 12, and 13.*

Experiment	*A*mo[#]	*K*m	*V*	*K*ip[#]	*R*ep[#]	*C*ap[#]
Supernatant fraction of LC tissue of No. 11						
1 Control	18.3	2.3	33.6	184	7.85	12.0
+ Fucoidan	12.3	4.1	39.8	268	13.5	12.9
2 Control	16.1	3.0	38.3	270	13.1	14.5
+ Fucoidan	12.3	4.0	39.2	406	20.1	15.7
3 Control	8.99	6.7	47.6	351	21.2	13.8
+ Fucoidan	8.30	7.6	50.0	618	39.1	18.0
Supernatant fraction of LC tissue of No. 12						
1 Control	16.7	8.3	109	416	57.4	31.0
+ Fucoidan	16.4	7.6	98	680	84.5	37.2
2 Control	15.6	7.9	98	412	51.0	28.2
+ Fucoidan	11.9	11.8	111	567	79.6	30.8
Supernatant fraction of LC tissue of No. 13						
1 Control	23.1	5.6	102	309	39.9	30.4
+ Fucoidan	21.3	6.1	103	389	50.8	32.9

Units are the same as in Table II. Fucoidan was used at A final concentration of 200 μg/ml. Using fucoidan (n=6), [#]is significantly different from the control ($p<0.05$) by Wilcoxon test.

Table VI. *Effect of fucoidan on biotinidase kinetics of normal liver.*

Experiment	*A*mo	*K*m	*V*	*K*ip	*R*ep	*C*ap
Homogenate fraction of Control 3						
1 Control	6.64	8.47	44.4	243	13.7	9.53
+ Fucoidan**	8.15	5.75	37.0	229	10.7	9.35
Supernatant fraction of Control 3						
1 Control	5.97	8.00	37.7	298	14.2	9.22
+ Fucoidan **	5.84	7.35	33.9	322	13.8	8.98
2 Control	4.33	8.33	28.5	273	9.85	6.53
+ Fucoidan	6.26	5.95	29.4	308	11.5	8.47
Supernatant fraction of Control 5						
1 Control	3.92	29.4	90.9	Noncompetitive inhibition[#]		
+ Fucoidan	4.08	26.3	84.7	Noncompetitive inhibition[##]		

No specific effect of fucoidan on *K*ip was observed although sample numbers were not sufficient (although not non-parametric, paired *t*-test indicated no significant effect). **Fucoidan concentration in these two experiments was at 2,500 μg/ml (the others were 200 μg/ml). [#]34% inhibition by 0.5 mM biotin; [##]49% inhibition by 0.5 mM biotin.

counteracted the increased *K*ip of HCC biotinidase or not was tested. It was found that fucoidan had the expected effect of reducing the *K*ip values in the HCC tissues. Conversely, fucoidan activated LC biotinidase, *i.e.* biotinidase capacities (*K*ip, *R*ep and *C*ap) all significantly increased in the LC

Table VII. *Effect of fucoidan on biotinidase kinetics in HuH-6 hepatoma cells.*

	Biotinidase kinetics					
	*A*mo	*K*m	*V*	*K*ip	*R*ep	*C*ap
Without fucoidan (control) HuH-6 homogenate	8.57	4.63	31.3	349	13.8	10.9
With fucoidan (at 200 μg/ml) HuH-6 homogenate	7.64	4.76	28.7	257	9.35	8.45

Units are the same as in Table II. HuH-6 cells were cultured with fucoidan or without (control) for 2 days with no apparent difference.

Table VIII. *Increase of biotin in the HCC tissue in the DEN-treated LEW rat.*

Liver sample	Total biotin (μg/g)	Free biotin (μg/g)	Ratio of free biotin (Free/Total × 100)
Normal liver (Control)	3.19±0.273	0.571±0.242	17.3%
HCC liver	9.45±1.34	6.50±2.57	68.8%
LC liver	6.23±0.966	3.57±2.13	57.3%

Male LEW rats were treated with DEN according to the procedure as described in the Materials and Methods section. At 18 weeks (25 weeks of age), control and DEN-treated rats were killed, and biotin content in the liver was assessed by a recently published procedure (22). Three portions for normal liver and six portions for HCC and LC livers were excised and measured. Mean±SD are shown. Mann-Whitney's *U*-test for both total and free biotin: 5% significance between normal and LC or HCC, between LC and HCC, and 1% significance between LC and HCC, respectively.

tissues. The results indicate that fucoidan reduced the capacity of biotinidase in HCC, and conversely increased the capacity of biotinidase in LC. The vitamin biotin serves as a covalently bound coenzyme for four major carboxylases (1, 2) and a growth factor in tissue cells (23-25). In addition, conjugation of biotin to histones (DNA-binding proteins) may be mediated by biotinidase, *i.e.* biotinylation of histones may play a role in cell proliferation (24), gene silencing (26) and cellular response to DNA damage (27). Theoretically, fucoidan may reduce the biotin supply in HCC (reducing cellular growth in HCC), but it may increase the supply in LC (increasing cellular growth in LC) by changing the biotinidase kinetics inversely.

Other oligosaccharides such as L-fucose, chondroitin sulfate, hyaluronic acid and carrageenan failed to affect the biotinidase kinetics in HCC and LC tissues. Although heparin showed a similar kinetic effect, it seemed to have a

side-effect on normal livers (data not shown). The biological activity of fucoidan is thought to be due to the similarity in structure between fucoidan and fucosylated glycans of certain extracellular membrane glycoproteins such as selectin (28), antistasin (29), proacrosin (30), bindin (31), GMP-140 (32) and annexin II (33). It is possible that the putative fucoidan- and heparin-binding sites are in a similar region of biotinidase. In support of this suggestion is the previous report that fucoidan and heparin bind to a similar region of annexin II (33). Taken together, the effect of fucoidan is probably mediated by a lectin-polysaccharide type of interaction with biotinidase.

As expected, fucoidan in the DMEM reduced the biotinidase capacities in the hepatoma HuH-6 cells (Table VII). Even at a concentration of 200 μg/ml, no cytotoxic effect from fucoidan was observed morphologically. In support of this finding is the fact that the concentration of albumin was unchanged by treatment with fucoidan (data not shown). The effect of fucoidan on the biotinidase kinetics of normal livers was tested; although not sufficient for statistical analysis, fucoidan did not seem to affect the normal livers.

The present study suggests that fucoidan has the potential to cure HCC without apparent side-effects. The mechanism of this specific effect only on livers with cancer is difficult to understand at this time, hence further studies are necessary to elucidate the exact role of fucoidan in the pathogenesis of HCC.

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