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Fucoidan-dependent Increased Membrane Components in HepG2 Cells: Effect of Fucoidan Is Not Due to Gene Expression

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Abstract. *Background: The precise mechanism of the therapeutic effects of fucoidan (sulphated polyfucose) on cultured hepatocarcinoma HepG2 cells is as yet unclear. Materials and Methods: Protein components between fucoidan-treated and non-treated HepG2 cells were compared through a quantitative micro-sequencing method. Results: A dramatic and immediate increment of the membrane compartment and a decrement of RNA virus by fucoidan, as an effect of the Ishi-Mozuku (an edible brown seaweed Mozuku of Japan), are demonstrated. The ratio of membrane glycoproteins to total cellular proteins increases from 28.9% to 43.2% (1.5-fold), and the positive-sense single-stranded RNA viral proteins among the total cellular proteins decrease from 5.3% to 0.29% (18-fold), respectively, in response to 0.102 mg/ml fucoidan in HepG2 cells over three days' period. Conclusion: Fucoidan seems to retard the growth of HepG2 cells through membrane glycoprotein metabolism. Therefore, fucoidan could be expected to have a therapeutic effect on hepatocellular carcinoma.*

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

Abbreviations: EDC: l-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide; HbA: haemoglobin A; AFP: alpha-fetoprotein; PTH: phenylthio-hydantoin; SEC: size-exclusion chromatography; HCC: hepatocellular carcinoma; LC: liver cirrhosis; Hc: hepatocyte cell; PBC: primary biliary cirrhosis; HCV: hepatitis C virus; HIV: human immunodeficiency virus; NCBI: National Center for Biotechnology Information; BLAST: Basic Local Alignment Search Tool.

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Key Words: Direct micro-sequencing, proteomics, protein determination, invaded microbes, membrane protein, hydrophobicity, de-anchoring, liver cancer, HCC, HCV, HIV, viral proteins, Ishi-Mozuku, fucoidan.

Our research group has previously shown that covalent binding of the proteins to glass-fiber disk is very important for quantitative direct-microsequencing (1).

Direct-microsequencing using l-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC) (2) has been successfully applied to protein component analysis in the human bile and human pancreatic juice (3).

Thus, we have applied this method to analyze homogenates of liver tissues, and of liver cell lines. It has been found that cultured hepatoblastoma cells (HuH-6) and retinoblastoma cells (Y79) mainly contain histone H3.1 in their homogenates, but tissues of adult livers mainly contain haemoglobin A (HbA) and albumin (4). Immortalised cultured cell lines seem to produce augmented histone H3.1 (4), and also D-aspartic acid (5). Furthermore, HCC tissues of the livers of LEW rat show a 10-fold increase in free-form biotin, compared to corresponding LC tissues (6).

Furthermore, these lines of evidence have been clearly re-confirmed by separating the homogenate sample into the supernatant and membrane fractions by ultra-centrifugation (100,000 × *g*, 90 min, 4˚C). In the present article, we extend and apply the improved direct-microsequencing deciphering method on fetal liver cells of Hc and on hepato-carcinoma HepG2 cells. It has been confirmed that Hc cells also contain high amounts of histone H3.1 (5.5% (w/w) among the total cellular proteins). The high content of histone H3.1 in the HepG2 cells is not changed by fucoidan treatment. We report on changes in the metabolism of membrane glyco-proteins of HepG2 cells in response to added fucoidan in the culture medium at 0.102 mg/ml. We also discuss on the mechanism of retardation of growth of HepG2 cells by fucoidan.

Materials and Methods

Chemicals and reagents. Standard PTH-amino acids (20 amino acids were mixed), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbo-diimide (EDC), pyridine (biochemical grade), 4-vinylpyridine, tri-n-butylphosphine, acetone, chloroform, methanol, dimethylformamide (DMF), and trifluoroacetic acid (TFA) treated glass fiber disk were obtained from Wako (Osaka, Japan). Bovine serum albumin (BSA),

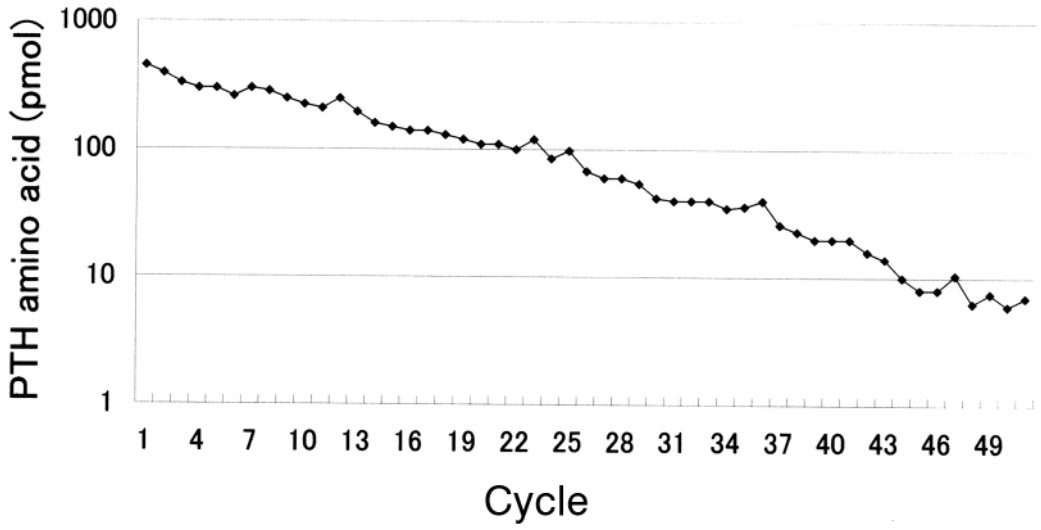

Figure 1. *Typical linear yield-line of PTH-amino acid obtained from the purified β-lactamase TEM fragment (analyzed from 24th to 76th amino acid residues of mature chain) of Escherichia coli O-157. A direct binding method with EDC was used. Hydrophobicity of this β-lactamase TEM fragment was considered to be relatively high (0.552), and this fragment was considered to be the membrane protein. Correction by the yield of PTH-amino acid was performed using Table I.*

non-edible fucoidan (from brown seaweed Mozuku (*Fucus vesiculosus*) of Ireland), and diethylnitrosamine (DEN) were purchased from Sigma (St. Louis, MO, USA). 3-Aminopropyltriethoxy-silane was from Shin-Etsu Chemical, Tokyo, Japan. Fetal calf serum (FCS) was from Moregate BioTech (Blimba, Australia). Chromatographically purified β-lactamase TEM fragment (from 24- of mature chain) of *Escherichia coli* O-157 was kindly provided by Dr. Manabu Tagawa (Department of Gastroenterology, National Research Institute for Child Health and Development; Now The Takeda General Hospital, Aidzu-wakamatsu-city, Fukushima, Japan) (Figure 1). The hepatoblastoma cell (HuH-6; from pediatric hepatoblastoma, 1-year-old Japanese male baby), the Japanese hepatoma cell (HuH-7; from HCC liver of 57-years-old male), and the American hepatoma cell (HepG2; from HCC liver of 15-years-old black male) were kindly provided by Rikagaku Kenkyusho (Tsukuba, Japan). Normal human fetal-hepatocyte cells (Hc) were kindly provided by Graduate School of Health Sciences, Gunma University (Maebashi, Japan).

Human liver autopsy and biopsy samples (HCC and corresponding non-tumorous tissues) were obtained from the Gunma University Hospital and the Nishi-Gunma Hospital as previously described (6). 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. Male LEW rats were purchased from Sankyo Labo Service Co., Tokyo, Japan, and tested for HCC as described previously (6). All the protocols for tests in rat were carried out in accordance with ethical guidelines for laboratory animals of National Research Institute for Child Health and Development.

Brown seaweeds of Ishi-Mozuku (*Sphaerotrichia divaricata*) and Kinu-Mozuku (Silky-Mozuku; *Nemacystis decipiens*) were purchased from Koh-Sushi Co., Anamizu-cho, Ishikawa, Japan. Edible fucoidans were extracted with acetic acid-water 1 : 2 (v/v), purified by 80% ethanol-aggregation method, and dried by us from these edible brown seaweeds Mozukus. Okinawa-fucoidan was prepared from the brown seaweed Okinawa-Mozuku (Futo-Mozuku; *Cladosiphon okamuraus*) as previously described (7). This Okinawa-fucoidan (containing glucose) surely inhibited the biotinidase activity (*K*ip) in the cell-free homogenates of liver HCC tissues. However, it turned out that this glycan did not inhibit the growth of the adults' HuH-7 and HepG2 cells at all, but inhibited the growth of the baby's HuH-6 cells. Therefore, this fucose-polymer glycan (containing glucose) was re-named as "Okinawa-fucoidan" in this text, since chemical structures seemed to be different from the fucoidan (pure sulphated poly-fucose).

Cell culture and preparation. Cell cultures were performed on usual non-coated plastic plates for HuH-7, and other cells were cultured on collagen-coated plates. Fucoidan from Ishi-Mozuku (*Sphaerotrichia divaricata*) was used at 0.102 mg/mL. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), streptomycin (100 U/mL) and kanamycin (100 U/mL) in a humidified 37˚C/5% CO_2 incubator. In order to compare suitably or reproducibly, cells were harvested after 3-days of culture (late-log-phase) with cell scraper (Corning), and obtained cells washed three-times with phosphate buffered saline (PBS) and cell volume was measured. Our previous work indicated that the gene expression was very sensitive to environmental nutrition and cell division cycle or growth phase (8), therefore we decided to compare as simple as possible in order to elucidate the differences occurred between normal and cancer state. Thus, elucidation was performed by the comparison of different specimens as far as we could. Washed cells were stored in liquid nitrogen at -196˚C until use.

Direct-microsequencing. Homogenates of HepG2 and other liver cell lines were prepared as previously described (4), and were ultra-centrifuged at 100,000 ×*g* for 90 min at 4˚C (Beckman L8-M Ultracentrifuge; Rotor Type 35). Obtained membrane fraction and

Table I. *Calculation method for each PTH-amino acid derived from the PPSQ-21A protein sequencer (Shimadzu)*.*

PTH-amino acid	True amount of amino acid in proteins (pmol) Factor
Asp (D)	measured (pmol) × 8.7
Glu (E)	measured (pmol) × 6.0
Asn (N)**	measured (pmol) × 7.8
Gln (Q)**	measured (pmol) × 4.6
Ser (S)	measured (pmol) × 20.7
Thr (T)**	measured (pmol) × 10.9
His (H)	measured (pmol) × 6.1
Gly (G)	measured (pmol) × 5.1
Ala (A)	measured (pmol) × 3.4
Tyr (Y)	measured (pmol) × 3.7
Arg (R)	measured (pmol) × 8.5
Cys (C)	measured (pmol) × 11.0
Met (M)	measured (pmol) × 6.2
Val (V)	measured (pmol) × 6.0
Pro (P)	measured (pmol) × 5.1
Trp (W)	measured (pmol) × 4.3
Phe (F)	measured (pmol) × 3.7
Lys (K)	measured (pmol) × 4.0
Ile (I)	measured (pmol) × 2.8
Leu (L)	measured (pmol) × 3.6

*pmols PTH-amino acid are calculated at attenuation 3. **15% of Asn (N) becomes to Asp (D), and 9% of Gln (Q) to Glu (E). If Gln or Asn is a major amino acid component, correction is performed. 50% of PTH-Thr becomes to PTH-ΔT (5-ethylidene-3-phenyl-2-thio-hydantoin).

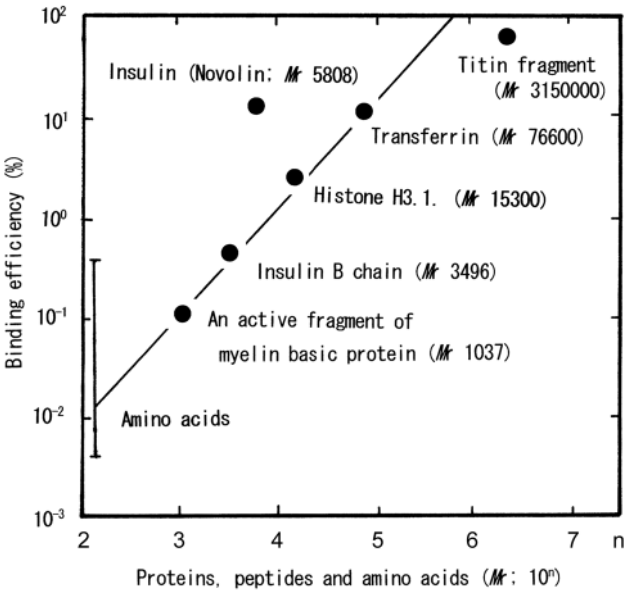

Figure 2. *Dependence of binding efficiency in the EDC-binding method upon the relative molecular mass. Binding efficiency of representative protein was expressed in %. A linear correlation line was shown as in the log-log plot.*

supernatant fraction were stored in aliquots at -196˚C until use. Protein determinations of membrane- and supernatant-fractions were performed by our SEC-HPLC protein determination method (3, 4, 10, 11).

Direct-microsequencing was performed three-times by using both of these supernatant- and membrane-fractions (0.08 mg of protein) as described previously (4) by using a PPSQ-2lA protein sequencer (Shimadzu, Kyoto, Japan). Proteins are bound covalently to the glass filters with EDC according to the method of Salnikow *et al*. (2), and cysteine as PEC is measured automatically (9). Reproducibility of production of PTH-amino acids is essential, and soluble cellular and membrane samples, which were stored at -196˚C, are found to be important for high binding-rate. If fresh sample is used, the amount of total identified proteins usually becomes 100±10% the amount of proteins, as determined independently by our SEC-HPLC protein-determination method. Reproducibility of this method depends only on the appeared PTH-amino acids, and direct-microsequencing is repeated three times. Then, the mean values are used for the next deciphering algorism (this algorism requires usually two weeks for the supernatant sample, and further two weeks for the membrane sample manually). Thus, a computer-aided software program of this deciphering algorism is expected to be deviced in order to obtain the analytical result promptly.

Determination of liberated PTH-amino acids is performed with the correction factors as shown in Table I. These correction factors are unique to the individual Shimadzu PPSQ-2lA protein sequencer. Trial to increase of the yield (or factors) of Ser and Thr by using HCl instead of TFA is not yet possible, since production of NaCl sometimes deteriorates the sequencer machine. If another protein sequencer is used, the correction Table I may again be changed.

Binding efficiency of various standard proteins by EDC is estimated, and is found to be dependent on the logarithm of the MW of proteins and peptides (Figure 2). Therefore, we used the correction factors in the protein determination process, using Figure 2, as follows; factor 1 for MW larger than 250,000, factor 2 for MW 40,000-250,000, factor 10 for MW 15,000-40,000, factor 50 for MW 5,000-15,000, factor 200 for MW 1,500-5,000, factor 714 for MW less than 1,500, respectively. If comparison of two samples of protein expression is necessary, standardization is performed.

Increase of PTH-amino acid (pmol of amino acids) of nth cycle is obtained by comparing to the previous (n–l)th cycle, these increments were scored. The amount of liberated amino acids from 1st to 20th cycle are usually determined and scored on a paper. Then, corrected estimations for the successive amino acid sequence (*e.g*. A-A-A-A or D-D-D-D *etc*.) are performed by comparing amount of pmol at (n+2) th, (n+1) th, n th, and (n–l) th cycles for each amino acid; *i.e*., when most high values are observed at the (n+2) th and the (n–1) th cycle, the second high value is displaced into the (n+1) th and the n th cycles.

Deciphering algorithm method. Firstly hemoglobin-alpha and -beta (HbA) were calculated in the case of human serum and tissue or rat tissue samples, and subtracted from the scored table. Since cultured cells except for fetal liver cells (Hc) did not contain HbA, this step was omitted. Then, in the case of liver tissues and liver cell lines, ceruloplasmin [precursor (1-), mature (19-), and fragment (214-)] and transferrin [precursor (1-), mature (19-)] were calculated and subtracted. The reason why the fragment of ceruloplasmin (from

Table II. *Proteins of Hc cells*.

Proteins	Amount (μg/mg of protein homogenate)
Isovaleryl-CoA dehydratase, mitochondrial (Arabidopsis thaliana (thale cress); 303-)	*28.6*
50S Ribosomal protein L4 (Caulobacter vibrioides; 195-212)	18.2
Lipoic acid synthetase (Lesionella pneumophila; 1-)	*17.6*
Genome polyprotein (Echovirus 9; N-myristoylated; 1139-)*	17.0
Minor capsid protein L2 (Human papillomavirus type 56; HPV; 441-464)##	10.6
UPF0336 protein MAP_4107 (Mycobacterium avium; 138-159)	*9.8*
Nuclease sbcCD subunit C (Treponeme pallidum; 1010-1047)	*8.6*
Arginine N-succinyltransferase (Yersinia enterocolitica; 274-)	*8.1*
Outer capsid protein VP5 (Broadhaven virus; 465-480) #	8.1
Histidyl-tRNA synthetase (Rickettsia canadensis; 74-)	*7.9*
*Genome polyprotein (HCV; S-palmitoylated and anchored state; Mr 250,000;766-)**	*7.7*
Genome polyprotein (Dengue virus 92; non-anchored; 583-)*	7.6
Tetraacyldisaccharide 4'-kinase (Pseudomonas aeruginosa PA7; 47-)	*6.5*
NADH dehydrogenase I subunit K (*Mycobacterium tuberculosis*; 52-)	6.3
UvrABC system protein C (Mycoplasma agalactiae; 320-)	*5.8*
Arginyl-tRNA synthetase (Mycobacterium tuberculosis; 298-)	*5.7*
Biotin synthetase (*Arabidopsis thaliana* (thale cress); 1-)	3.5
Envelope glycoprotein gp160 (HIV-2; retrovirus; palmitoylated; 204-)*	3.4
FHS 1 (Streptococcus pyogenes; 20-)	*2.5*
DNA polymerase III subunit beta (Mycoplasma capricolum; 220-)	*1.8*
RNA polymerase subunit beta (Streptococcus mutans; 664-)	*1.2*
Total	186

*Viral, bacterial and plant proteins are present at 18.6%. The amount of viral proteins in Hc cells (4.7%) is similar to HepG2 cells (6.6%). Traffic accident might have introduced the proteins (or genes) of plant proteins (indicated in bold letter). Viral proteins are indicated in red. Membrane proteins are indicated in italics. Cysteine residues at 1972 and 1976 of genome polyprotein (*HCV*) are S-palmitoylated. Cysteine residue at 771 of envelope glycoprotein gp160 (*HIV-2*) is only S-palmitoylated at one site, hydrophobicity is 0.54, and it may come from the soluble fraction. # ds RNA virus. ## ds DNA virus. * +ss RNA virus.

214-) is always detected was not yet fully-understood at this time. Then, albumin, albumin-precursor, alpha-fetoprotein (AFP) and AFP-precursor were calculated and subtracted. The major protein of rat skeletal muscle is unexpectedly found to be rat albumin, therefore it should be subtracted in this case. In the case of cultured cells, biotin synthetase of bacterial sequences (*Bacillus subtilis* or *Helicobacter pylori*), and lipoic acid synthetase of *Bacillus subtilis*'s or human sequence were detected, and calculated to subtract. Then, sequences of our human serum biotinidase (DQPGEVAVADVAQVY-) (12) and human urine (or kidney) biotinidase (our unpublished result; *i.e.*, AVPPQVGNGQEGQTN-) were calculated and subtracted. In the case of the N-terminal portion of the human serum biotinidase, two or three amino acids were observable at the same position; *i.e.*, in 1st(A, K, S), 2nd (N, H), 3rd (D, N), 5th(D), 6th(P), 12th (E, F), 13th (F, L), 14th (A) of amino acid residues (12). In these cases, amount of amino acids was divided according to the amount of amino acid present, and subtracted from these amino acids in the table. The sequence of milk-type biotinidase (our unpublished result; FPSYVAVTKV VPPYG-) was not detected in the serum, but appeared in the fetal liver cells (Hc cells). The sequence of human serum biotinidase reportedly by Cole *et al.* (13) (AHTGEESVADHHEAE-) only appeared in the fetal liver cells (Hc cells) and the serum of the inflammatory patients. Therefore, this sequence as reported by Cole *et al.* may be re-named as the fetal-biotinidase or the inflammatory-biotinidase. However, the following database searches could not be continued at all without using these biotinidase sequences, since biotinidase was one of major component proteins in the liver and serum in humans.

Then, the remaining amino acid amounts were scored again in table format. The major amino acid sequence of 14 digits from the table (from 3rd to 16th) was then searched for by using the protein database tool of Protein BLAST (Basic Local Alignment Search Tool) of NCBI (National Center for Biotechnology Information) at the URL http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome

If several high-score amino acids were present at the same position, a random search was performed in the case of two amino acids. If more than two amino acids were present, then redundant sequences of same amino acids were tested. Proteins showing more than 6 matched digits were chosen, and were included on the other table. After referring the database, sequences were extended to indicate 1st to 16th digits. Usually a higher than 90% protein matching was considered to be highly possible to be present in the sample, and the mol amount of the protein or protein fragment was calculated. If the database search was suddenly stopped, search on viral and bacterial proteins was subsequently performed. Since antibiotics against bacteria were present in the culture medium, established cultured cells usually contained no invaded bacterial proteins. Then, matching to a viral protein usually occurred, and this viral protein was chosen. The appeared amount (pmol) of matched amino acid were then plotted on the semi-logarithmic graph sheet,

Table III. *Proteins of Hc cells**.

Proteins (cellular proteins; 81.4 %)	Amount (μg /mg of protein homogenate)
Histone H 3.1. (1-)	55.2
Protein kinase C eta type (639-683)	47.5
Actinin-associated LIM protein (36-)	33.7
Protein FAM8A1 (293-)	32.5
Dyslexia susceptibility 1 candidate gene 1 protein (402-420)	31.3
Olfactory receptor 56A3 (138-)	28.6
Protein regulator of cytokinesis 1 (502-)	23.9
Desmoyokin (3712-)	23.5
Cytochrome c oxidase subunit 2 (207-227)	21.9
Dyslexia susceptibility 1 candidate gene 1 protein (402-420)	21.9
Mitochondrial chaperon BCS1 (407-419)	*19.8*
Early activation antigen CD69 (159-199)	19.1
Protein SSX8 (75-)	18.6
RWD domain-containing protein 2A (178-)	18.6
Neutrophil NADPH oxidase factor 4 (18-)	17.1
Histone deacetylase 4 (HD4; 960-)	16.7
Notch homolog 2 N-terminal-like protein (207-236)	15.8
Protein SCD6 homolog (441-463)	15.5
Brain-specific angiogenesis inhibitor 2 (159-)	15.2
Glutathione S-transferase A4 (208-222)	15.2
Chymase (137-)	14.4
Protein janus-A homolog (1-)	*14.3*
Uncharacterized protein ENSP00000373828 (18-)	14.0
T-cell-specific kinase (493-)	*11.4*
Relaxin receptor 2 (731-754)	10.6
Jumonji domain-containing protein 2B (765-)	10.5
Protein CDM (157-)	*10.2*
Dihydropteridine reductase (63-)	9.8
Modulator recognition factor 2 (1011-)	9.5
Plancental-specific 1-like protein (140-158)	9.5
Protocadherin alpha-C2 (127-)	9.4
Uveal autoantigen with coiled-coil domains and ankyrin repeats (573)	9.1
OB DNA-binding domain-containing protein C16orf175 (88-)	8.4
Transcription factor Sp5 (380-398)	*8.1*
LON peptidase N-terminal domain and RING finger protein 1 (69-)	*8.0*
Leucine-rich repeat-containing protein 40 (137-)	7.6
Lariat debranching enzyme (513-544)	*7.1*
MEK kinase 5 (341-)	6.7
Proton myo-inositol cotransporter (36-)	6.4
Metalloreductase STEAP2 (435-)	*6.3*
Nuclear protein ZAP3 (581-)	*6.3*
Neurexin-2-alpha (579-)	6.1
Uncharacterized protein C9orf79 (151-)	*6.0*
Zinc finger protein LOC400713 (390-)	6.0
Syntaxin-7 (46-)	5.7
Chromatin-helicase-DNA-binding protein 6 (251-)	*5.7*
Protein C (20-)	5.5
Erasin (282-)	*5.3*
Glutamate decarboxylase (548-)	*5.3*
Lectomedin-2 (496-)	5.3

*Proteins from originating membrane fractions are indicated in italics. Titin is a serum marker of adulthood (our unpublished observation); *i.e.* titin is undetectable in Hc (from fetal livers), but detectable in HepG2 (from male HCC liver of a 15 years old patient) (Tables IV and V).

and the linear line was obtained (Figure 1). Using this line, the amount of protein (pmol) was estimated. The N-Terminal sequence of immunoglobulin G (IgG) is variable (variable region; 1-126 of mature chain), and the database was not yet fully-available.

But, the result of searches was essentially similar, since immunoglobulins in most the biological samples, except serum, were minor components. The consideration for the presence of c.a. 15% of IgG may be necessary in the case of serum samples. Then, relative

Table IV. *Proteins of Hc cells**.

Proteins (cellular proteins; 81.4%)	Amount (μg/mg of homogenate protein)
Transferrin (TF; 1-)	5.3
Sodium/hydrogen exchanger 11 (850-)	*5.1*
Melanocortin receptor 5 (147-)	4.8
PNMA-like protein 2 (194-)	4.8
SEN1 homolog (2237-)	4.8
Prenylcysteine oxidase 1 (337-)	4.5
Collagen alpha-5 (VI) chain (1383-)	*4.4*
Lipase member I (327-)	4.3
Lipoic acid synthetase, mitochondrial (Human; 1-)	4.0
Aquaporin-11 (55-)	*3.9*
Hemoglobin alpha chain (1- of mature)	3.7
Leucine-rich repeat serine/threonine-protein kinase 1 (488-)	*3.6*
Retinoic acid-induced gene G protein (180-)	*3.6*
Drosophila Delta homolog 4 (43-)	3.5
Coiled-coil and C2 domain-containing protein 1B (180-)	*3.2*
Transferrin precursor (TFpre; 1-)	*3.2*
Elongation factor 1-beta (91-)	*3.1*
Biotin synthetase (Bacillus subtilis; 1-)	*3.1*
Ceruloplasmin (from 214- of pre)	2.7
Biotin synthetase (Helicobacter pylori J99; 1-)	*2.6*
Poly [ADP-ribose] polymerase 12 (22-)	*2.5*
Synaptic vesicle glycoprotein 2B (262-)	2.3
Tripartite motif-containing protein 42 (75-)	*2.3*
Serum biotinidase (our sequence; 1-)[12]	2.1
Kynurenine 3-monooxygenase (7-)	*2.1*
Kinesin light chain 4 (161-)	*2.1*
Hemoglobin beta chain (1- of mature)	*1.9*
Milk biotinidase (1-; our unpublished sequence)	1.9
Urine biotinidase (1-; our unpublished sequence)	1.9
alpha-Fetoprotein (mature chain; from 1-)	*1.6*
Transformation-related gene 15 protein (333-)	*1.6*
SLIT and NTRK-like protein 1 (76-)	*1.5*
alpha-Fetoprotein precursor (from 1-)	*1.1*
Flamingo homolog 1 (2872-)	*1.0*
Fetal-type biotinidase (1-) [13]	0.3
Albumin (1-)	0.0
Albumin precursor (1-)	0.0
Ceruloplasmin (from 19- of precursor)	0.0
Ceruloplasmin precursor (from 1- of precursor)	0.0
Total	814

*Amino acid sequence of the putative human fetal-biotin-synthetase has not yet been available. Sequences of human milk biotinidase milk biotinidase was isolated from a mixture of human milks provided by several lactating females and fetal/inflammatory biotinidase (NCBI, PubMed; UniProtKB/Swiss-Prot: P43251.2) were FPSYVAVTKVVPPYG- (1st V, 7th N, and 11th I was also detected as assessed by direct-microsequecing) and AHTGEESVADHHEAE-, respectively. Proteins coming to the membrane fractions are indicated in italics.

molecular mass (MW) of protein or fragment of protein matching was calculated, and corrected by the binding factor according to the MW. Then, we created a table in a work sheet of Microsoft Excel. If 8 digits of search were not possible to continue, this step was the termination point in the direct-microsequencing-deciphering algorithm search. Usually, 40-times of sequence searches of 8 digits were performed, and about 120 proteins and/or protein fragments were identified in the supernatant or membrane fraction of livers and cultured liver cell lines. About 70% of proteins were N-terminal closed in eukaryotic cells (14), but only 5% (Q among 20 amino acids) might be strongly closed by pyroglutamic acid. Therefore, each supernatant- or membrane-fraction seemed to contain approximately 125 proteins; 120/(1-0.05)=126 species of protein molecules. Thus, approximately 250 species of proteins seemed to be present in the human livers and cultured liver cell lines.

Protein hydrophibicity. Hydrophobicity of the proteins is defined as; Hydrophobicity=1 – Hydrophilicity. Hydrophilicity was defined as

Table V. *Viral proteins of HepG2 cells without addition of fucoidan.1*

Proteins (Viral proteins 6.6 %)	Amount (μg /mg of protein homogenate)
Genome polyprotein (HGV; GB virus C; 926-)*	14.0 (may be de-anchored)
*Genome polyprotein (HCV; hydrophobic of 0.586; Mr 250,000; 821-)**	*7.2 (may be de-anchored)*
Spike glycoprotein (Human coronavirus 229E; 173-)*	7.1
Envelope glycoprotein gp160	
*(HIV-1; retrovirus; may be de-palmitoylated and hydrophilic of 0.554; 454-)**	*5.7 (may be de-anchored)*
Gag-Pol polyprotein (HIV-1; retrovirus; N-myristoylated; 723-)*	5.1
Genome polyprotein (Human poliovirus=Human coxsackievirus; N-myristoylated; 1562-)*	4.7
Protein UL50 (Human cytomegalovirus; HHV-5; 264-)##	2.5
*Spike glycoprotein (Human coronavirus; 523-)**	*2.1*
Helicase/primase complex protein (Varicella-Zoster virus; HHV-3; 839-)##	1.8
*Envelope glycoprotein gp160 (HIV-1; retrovirus; may be de-palmitoylated but hydrophobic; 344-)**	*1.7 (may be deanchored)*
Protein C10 (Variola virus=Vaccinia virus; 90-)##	1.7
*Protein L (Human respiratory syncytial virus; HRSV; 1668-)**	*1.6*
*Hemagglutinin-neuraminidase (Newcastle disease virus; 114-)***	*1.5*
Structural glycoprotein gp41 (Autographa californica nucear polyhedrosis virus; 230-)##	1.3 (vaculovirus of insect)
Protein F12 (Variola virus=Vaccinia virus; 294-)##	*1.1*
Protein Rev (HIV-1; retrovirus; 7-)*	1.0
Latent membrane protein (Epstein-Barr virus; EBV; HHV-4; 136-)##	*0.9*
Envelope glycoprotein gp160	
*(HIV-1; retrovirus; may be de-palmitoylated and hydrophobic, but pI 10.4; 779-)**	*0.8 (may be deanchored)*
Replication protein E1 (Human papillomavirus type 60; HPV, 362-) ## 0.8	
RNA-directed RNA polymerase (Infectious bursal disease virus; IBDV; 432-)#	*0.7*
Neuraminidase (Influenza A virus; 389-)**	0.6
*Protein Nef (HIV-2; retrovirus; 87-)**	*0.6*
Envelope glycoprotein gp95	
*(Rous sarcoma virus; retrovirus; may be de-palmitoylated but hydrophobic of 0.604; 462-)**	*0.5 (may be deanchored)*
*Protein alpha (Nodamura virus; Nihon-nohen-virus; 262-)**	*0.4*
VETF large subunit (Variola virus; Vaccinia virus; 668-)##	0.3
Protein B15 (Variola virus=Vaccinia virus; 109-)##	0.3
Late L1 52 kDa protein (Human adenovirus; 10mer; 364-)##	0.2
*Neuraminidase (Influenza A virus; 18-)***	*0.1*
Uncharacterized protein L330 (Mimivirus; 238-)##	*0.1*
Total	66

Proteins of retrovirus are indicated as red letters, HCV and HGV proteins are in blue. RNA viruses are present at 83.3% among viruses. Retroviruses are present at 23.3% among viruses, and *HCV* and *HGV* at 32.1%. Membrane proteins were indicated in italics. Cysteine residues at 769 and 842 of envelope glycoprotein gp160 (HIV-1) are S-palmitoylated. 79% (1.7/(1.7+5.7+0.8)=0.79) of the envelope glycoprotein gp160 (*HIV-1*) is deanchored state. Cysteine residues at 1972 and 1976 of genome polyprotein (*HCV*) are S-palmitoylated, but this protein fragment in this table (821-) is hydrophobic (0.586) and is sufficient to be present at the membrane fraction. Cysteine residues at 569 and 572 of envelope glycoprotein gp95 (Rous sarcoma virus) are S-palmitoylated, but this protein fragment in this table (462-) is hydrophobic (0.604) and is sufficient to be present at the membrane fraction. # ds RNA virus. ## ds DNA virus. * + ssRNA virus. ** –ss RNA virus.

follows: (Asx + Thr + Ser + Glx + Lys + His + Arg)/(total amino acid) (15). Hydrophobicity larger than the value of 0.55 is tentatively considered to be the hydrophobic membrane protein in this text, since this hydrophobicity value of Reference 15 is well-coincided to the definition of the membrane protein as determined by the ultracentrifugation method; *i.e.*, membranes were defined as the cellular components precipitated at 100,000 × *g* for 90 min at 4˚C.

Determination of lipoic acid (thioctic acid; lipoate). The amount of total lipoic acid (thioctic acid) in rat livers were determined by a newly-developed high-performance affinity liquid chromatography (16).

Statistics. Since the numbers of the data to be compared were insufficient for estimating the distribution pattern, non-parametric analysis was applied in this text. Therefore, values are indicated as the median and range. Non-parametric statistical analysis was performed according to Reference 17.

Results and Discussion

We first constructed a correction table to estimate the amount of amino acids present in the protein sample from the provided PTH-amino acid (pmol) reading of the machine (Table I). Degradation of Ser and Thr during micro-sequencing was indeed large as expected by the use of TFA (Table I), however such a good yield line was obtainable following correction factors in Table I, using purified

Table VI. *Viral proteins of HepG2 cells treated with fucoidan for 3 days*[1].

Proteins (Viral proteins; 4.6%)	Amount (μg/mg of homogenate protein)
Glycoprotein H (*Bovine* herpesvirus type 1.1 (11-)##	17.3
Latent membrane protein 2 (EBV= Human herpesvirus 4; HHV-4; 207-)##	*7.5*
Cell fusion protein (Human herpesvirus 1; HHV-1; 99-)##	*3.3*
*Protein L (Mumps virus; 1399-)***	*2.3*
Ribonucleotide reductase (HCMV=Human herpesvirus 5; HHV-5; 159-)##	*2.0*
*Genome polyprotein (HCV; fragment but hydrophobic; Mr 83,000; 2279-)**	*2.0*
Uncharacterized protein L515 (Mimivirus; 122-)##	*1.6*
Putative serine/threonine-protein kinase/receptor R181 (Mimivirus; 1392-) ##	1.3
Nucleoprotein (Influenza C virus; 365-)**	1.0
DNA polymerase (Human adenovirus type 40; 1002-)##	1.0
Capsid assembly protein U30 (Human herpesvirus 6: HHV-6; 757-)##	*0.9*
*Protein Nef (HIV-1; retrovirus; 45-)**	*0.9*
Uncharacterized protein L237 (Mimivirus; 11-)##	*0.7*
Serpin-1 (Variola virus= Vaccinia virus; 113-)##	*0.7*
Uncharacterized protein L172 (Mimivirus; 150-)##	*0.7*
*Hemagglutinin (Influenza A virus; 394-)***	*0.5*
Fiber protein (Human adenovirus type 12; 436-)##	*0.4*
U10 protein (Human herpesvirus 7; HHV-7; 340-)##	*0.3*
*Non-structural protein 4b (Human coronavirus 229E; 1-)**	*0.2*
Capsid protein VP26 (EBV= Human herpesvirus 4; HHV-4; 103-)##	*0.2*
Uncharacterized protein L43 (Mimivirus; 147-)##	*0.2*
Regulatory protein E2 (Human papillomavirus type 2a; HPV; 339-)##	*0.1*
Minor capsid protein L2 (Human papillomavirus type 67; HPV; 430-)##	*0.1*
Viral cathepsin (Orgyia pseudotsugata NPV; OpNPV; 305-)##	*0.1 (vaculovirus)*
RNA helicase I 8 (Variola virus=Vaccinia virus; 15mer; 662-)##	*0.04*
Total	46

[1]Proteins of retrovirus are indicated as red letters, and HCV and HGV were in blue. Fucoidan from Ishi-Modzuku was added at 0.102 mg (dry weight)/mL DMEM medium for 3 days. Retroviral proteins are dramatically reduced from 23.3% to 2.0% (11.7-fold) among viral proteins by the treatment with fucoidan for 3 days. RNA viruses are present at 15.0 % among viruses. Membrane proteins are indicated in italics. Cysteine residues at 1972 and 1976 of genome polyprotein (*HCV*) are S-palmitoylated, but this protein fragment in this table (RNA-directed RNA polymerase portion; RdRp) may not contain palmitic acid, but hydrophobicity (0.565) of this fragment is sufficient to be present at the membrane fraction.
ds RNA virus. ## ds DNA virus. * +ss RNA virus. **–ss RNA virus.

β-lactamase TEM fragment (from 24- of mature chain) of *Escherichia coli O-157* (Figure 1). The binding efficiency of proteins by the EDC method was logarithmically-depended upon the MW of the proteins and peptides as shown in Figure 2. Therefore, we corrected the protein amount by using this log-log line of Figure 2 (correction factors are shown in the Materials and Methods section).

The component proteins of Hc cells (fetal liver cultured cells) were determined, and are presented in Table II (viral and bacterial proteins) and Tables III and IV (cellular proteins).

The component proteins of HepG2 (derived from an HCC liver of 15-year-old male from the USA) were also determined and are summarized in Tables V and VI (viral proteins with and without treatment of fucoidan), Tables VII-IX (cellular proteins; without treatment of fucoidan) and Tables X-XIII (cellular proteins; with treatment of fucoidan for 3 days).

Fetal cultured Hc liver cells contained 19% of the invaded microbes (virus, bacteria, and plant) (Table II), which was of similar level to human breast milk (16%; virus, bacteria, and yeast) and to cow's milk (17%; virus, bacteria, and yeast) (data not shown). Interestingly, bacteria of *Lactobacillus casei (Shirota)* contained 12% of invaded bacteriophages (data not shown). Human biopsy tissues of hospitalized patients also showed similar results, but major virus and minor bacteria were detected; *i.e.*, pseudo-cancer of liver (non-cancer) contained 19%, the LC portion of the leprosy patient 15%, HCC portion of primary biliary cirrhosis (PBC) patient 30%, respectively, of the invaded microbes (virus and bacteria) among total tissue proteins (data not shown). It is also interesting that the DNAJ protein (Hsp40; Heat shock protein 40 kDa of *Mycobacterium leprae*) appeared only in the LC portion of the leprosy patient, which suggested that the sensitivity of detection power of proteomics was highly and sufficiently performed by this microsequencing method. The liver of the leprosy patient also contained the unique soil-related plant-tumor making bacterium *Agrobactrium*

tumefacience, and plant virus (*Yellow mosaic virus*) proteins, suggesting that co-infection of *Mycobacterium leprae*, *Agrobactrium tumefacience*, and plant virus (*Yellow mosaic virus*) in the patient might have occurred. Increased histone H3.1 in the LC portion of the liver of the leprosy patient has been also detected. High levels of total histone H3 transcripts (mRNA) in different tissues of plant *Alfalfa* has been reported showing a particularly high level in the somatic embryos (18). Thus, histone may be essential for immortality in plant cells and in human leprosy cells, but seemed not to be directly related to human cancer. Furthermore, the liver biopsy of HCC patient with *HCV* contained only viral proteins (LC tissue 12% and HCC tissue 16%, respectively, among the total tissue proteins), suggesting that antibiotics against the bacteria might have been used during hospitalisation of the patient. Fetal Hc liver cells contained 19% of invaded microbes; *i.e.*, 4.7% viral and 14% bacterial proteins (Table II). Since fetal Hc cells were prepared from the mixed fetal-livers obtained from the pregnant women who died of traffic accidents in USA, immediate infection by the invading microbes (virus, bacteria, and plant) might have occurred. These invaded microbes might still persistently have been remained in cultured Hc cells (few times of the passages of culture) in spite of the presence of antibiotics against bacteria in the culture medium. On the other hand, established hepatocarcinoma HepG2 cells were cultured for numerous passages with antibiotics, and bacterial proteins fully disappeared (Table V). Thus, the content of viral proteins in Hc cells (4.7%; Table II) and in HepG2 cells (4.6%, Table VI, cultured with fucoidan) finally returned similar values. However, the viral content of HepG2 cells without fucoidan treatment was considerably higher (6.6%, Table V). The median viral content in the serum of non-cancer persons was 3.1% (n=9; range 0.4-4.5%) (19). Biopsies of livers of three HCC patients and a pseudo-cancer patient contained very high virus content, of 13.5% (n=5; range 8.1-22.2%), although the data of the virus content in the non-cancer and non-inflammatory healthy livers were not obtainable. Therefore, the metastasis of cancer might easily occur by infection or diffusion of virus to other organs (20). Viral protein content in washed cells and serum, higher than 5% among the total cellular proteins might be an indicator for "the state of cancer". However, prompt determination of viral content may be difficult using the microsequencing method, which requires for analysis time of about one month using the presently used manual microsequencing algorithm.

HCV genome polyprotein is an anchored membrane glycoprotein (21, 22), and is detectable in the membrane fractions of cultured cells of Hc and HepG2, and of livers of diseases (n=4). Non-cancer fetal Hc cells contain 7.7 μg (0.77%; MW 250,000) HCV genome polyprotein (Table II), which may not be deanchored. Its level in hepatocarcinoma HepG2 cells is 7.2 μg/mg (0.72%; MW 250,000; Table V), but this may be deanchored. Fucoidan seems to reduce it to 2.0 μg/mg (0.20%; MW 83,000; Table VI), and the protein may not be of the deanchored state. The presence of HCV genome polyprotein in the liver tissues is also observed; *i.e.*, pseudo-liver-cancer 0.44% (membrane MW 130,000), LC tissue of leprosy 2.2% (membrane; MW 230,000), HCC tissue of PBC 3.5% (soluble; MW 230,000), LC tissue of a HCC with HCV 0.79% (soluble; MW 40,000) and HCC tissue of a HCC with *HCV* 0.55% (membrane; MW 340,000), respectively (data not shown). MW of HCV genome polyprotein in the non-cancer state tissues and the fucoidan-treated HepG2 cells seems smaller compared to the cancer state. Therefore, the presence of a large molecular mass HCV genome polyprotein in liver cells may be a risk factor for HCC or liver cancer with immortality.

Envelope glycoprotein gp160 (HIV) was dramatically reduced by fucoidan in HepG2; *i.e.*, from 8.2 μg/mg (0.82%; without fucoidan) to 0.0 μg/mg (0.0%; with fucoidan) (Tables V and VI). Although data in other hepatocarcinoma cells such as HuH-7 may also be necessary to derive a conclusion, fucoidan may be important for cancer cells to obtain the normal innate immune system by reducing HIV virus. Only a minor amount of the membrane protein Nef of HIV remained at the same levels (Table V; without fucoidan 0.6 μg/mg, and Table VI; with fucoidan 0.9 μg/mg) by fucoidan, however the HIV-1 Nef-interacting protein (T-complex protein 1 subunit eta; membrane protein made from the cellular DNA gene) is expressed in the fucoidan-treated HepG2 cells (Table X; 4.2 μg/mg). Therefore, the virulent viral Nef protein remaining in fucoidan-treated HepG2 cells may be neutralized by this cellular membrane protein (23). Fucoidan made from the edible Ishi-Mozuku at 0.102 mg/mL, dramatically reduces the proteins of plus-sense (+) single-stranded (ss) RNA virus from 5.3% to 0.29% (18.1-fold reduction) among the total cell-proteins (Table V and Table VI), while proteins in Hc cells are 3.6% (Table II). Hepatitis virus (*HCV* and *HGV*) plus retrovirus (*HIV* and *Rous Sarcoma* virus) are also reduced from 3.7% to 0.29% (12.6-fold reduction) among the total cell proteins (Tables V and VI), and those in the Hc cells is 1.1% (Table II). Reduction of retrovirus of *HIV* and *Rous Sarcoma* virus is also reduced from 2.3% to 0.2% (11.7-fold reduction) among the total cell proteins (Tables V and VI), and those in the Hc cells is 0.34% (Table II). HIV is a notorious virus responsible for human immunodeficiency (AIDS), and reduction of retroviral proteins seems to be essential for cure of HCC (24). Human papillomavirus (*HPV*) infection appears to be a necessary factor in the development of almost all cases of cervical cancer (25). Tumor formation in plants by the bacterium *Agrobacterium tumefaciens* has turned out to be caused by a symbiotic tumour-inducing (*Ti*) plasmid (or phage virus) (26). *Helicobacter pylori* infections have been linked to gastric adenocarcinoma (27), and the

Table VII. *Proteins of HepG2 cells without addition of fucoidan*.*

Proteins (Cellular proteins; 93.4 %)	Amount (μg/mg of protein homogenate)
Titin (17847-)	119
Titin (6505-)	*89.2*
Usher syndrome type-2A protein (Usherin; 448-)	34.4
Epiplakin (1029-)	29.3
ATPase family AAA domain-containing protein 2 (259-)	21.5
Protocadherin-16 (393-)	21.0
Serum (sBIN) biotinidase (1-)	16.4
Hornerin (852-)	14.7
Lipoamidase precursor (mLIP; CEL; BSSL; 4-)	14.0
Laminin alpha-2 chain (2185-)	13.4
Nardilysin (183-)	*12.9*
Insulin receptor substrate 2 (IRS-2; 368-)	*12.7*
Ceruloplasmin precursor (from 1-)	12.2
Ceruloplasmin (from 19- of pre)	12.0
Bifunctional protein NCOAT (Nuclear cytoplasmic O-GlcNAcase and acetyltransferase; 38-)	*11.5*
Lipoamidase (May be LIP/BIN; Enterococcus faecalis; 1-)	11.2
Complement C4-B (228-)	10.8
Albumin precursor (from 1-)	10.6
alpha-Fetoprotein precursor (from 1-)(mature chain is not present)	10.6
ATP-dependent helicase BTAF1 (372-)	10.6
Histone H 3.1. (2-)	10.6
Ceruloplasmin (from 214- of pre)	9.6
Myosin-10 (752-)	9.4
Histone-lysine N-methyltransferase, H3, Lys-9 specific 5 (12-)	8.9
von Willebrand factor-cleaving protease (ADAMTS-13; 244-)	8.3
Albumin (1-)	8.2
Jmj C domain-containing histone demethylation protein 3B (4-)	7.8
Transferrin precursor (TFpre; 1-)	7.8
Protein FAM21C (238-)	7.6
Fatty acid synthase (225-)	*7.6*
Bromodomain and WD repeat domain-containing protein 1 (1277-)	7.5
Sodium/hydrogen exchanger 1 (14-)	7.5
Fragile X E mental retardation syndrome protein (380-)	7.0
Heterogeneous nuclear ribonucleoprotein U (79-)	7.0
Kinesin-like protein KIF3B (389-)	6.7
Kinesin-like protein KIF3B (390-)	6.7
Macrophage ABC transporter (152-)	*6.6*
Macrophage mannose receptor 2 (597-)	6.4
Down syndrome cell adhesion molecule (31-)	*6.2*
Histone H 2A (2-)	6.0
Histone H 2B (2-)	5.8
Plexin-D1 (195-)	*5.7*
SAPS domain family member 1 (120-)	5.5
Vanilloid receptor-like protein 2 (107-)	5.5
Stereocilin (137-)	5.4
ADAMTS-19 (441-)	*5.2*
Human immunodeficiency virus type I enhancer-binding protein 2 (HIV-EP2; 1912-)	5.0
Myb-related protein B (21-)	4.9
Histone H 4 (2-)	4.7
Ankyrin repeat domain-containing protein 50 (741-)	4.6
Breast cancer anti-estrogen resistance 2 (577-)	4.5
Nuclear receptor coactivator 1 (92-)	*4.4*
Zinc finger protein 84 (130-)	4.4
Ryanodine receptor 1 (RyR1; Skeletal-muscle-type; 4427-)	*4.3*
Lipoic acid synthetase (Bacillus subtilis; 1-)	4.3
Structural maintenance of chromosome 2-like 1 protein (613-)	4.2
A-kinase anchor protein 8 (65-)	4.1
G2/mitotic-specific cyclin-F (208-)	4.1
Cold shock domain-containing protein E1 (236-)	4.1

Table VII. *Continued*

Table VII. *Continued*

Proteins (Cellular proteins; 93.4 %)	Amount (μg/mg of protein homogenate)
DAG kinase eta (Diglyceride kinase eta; 6-)	*4.0*
Far upstream element-binding protein 2 (167-)	3.9
Bardet-Biedl syndrome 10 protein (288-)	3.6
Excitatory amino acid transporter 1 (39-)	3.6
Glutaminyl-tRNA synthetase (272-)	3.6
Myelin transcription factor 1 (620-)	3.6
Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 (389-)	3.6
Protein kinase C-binding protein NELL2 (319-)	3.6
Transcription intermediary factor 1-gamma (64-)	*3.5*
Hepatocellular carcinoma-associated antigen 56 (Ligatin; 113-)	3.4

*Proteins of the membrane fractions are indicated in Italics. Titin is a serum marker of adulthood; *i.e.* titin is detectable in HepG2 (from male HCC liver of 15 years of age) but not detected in Hc (from fetal livers).

transformation of the gastric cancer cells may be speculated to have occurred by the plasmid or virus residing in this bacterium such as in the case of the tumour-inducing (Ti) plasmid residing in the plant bacterium *Agrobacterium tumefaciens* (26). Furthermore, virus or phage infection changes the surface glycochains of the dominated host cells (phage conversion), and prevents the invasion of other virus or phage to enter the conquered cell. Thus, glycochains of the cell surface may be changed by the invaded viral glycochain-converting enzymes (28). Therefore, the virus infection to the host cells may be an important issue for cancer and metastasis, and this glycochain conversion may be performed by the viral enzymes and viral genes but not by the cellular enzymes or cellular genes; *i.e.*, cellular gene expression may not be directly related to cancer differentiation. Fucoidan may directly prevent the infection from virus, and the possibility of direct inhibition of HIV reverse trascriptase activity by fucoidan *in vitro* has already been reported (29). Other DNA viral proteins seem to increase within the three days of fucoidan treatment; 11.0 μg/mg (without fucoidan) to 38.4 μg/mg (with fucoidan) (Tables V and VI). However, this level of DNA virus might also be reduced during the longer treatment time by fucoidan due to the slow-preventing effect against DNA virus; *i.e.*, oral administration of fucoidan for 3 weeks had been necessary in the mice cases (30). Following, fucoidan seems to dramatically reduce the viral proteins through direct inhibition of viral replication; *i.e.*, immediate inhibitions on viral protein synthesis and viral genome replication.

Comparative biochemical studies among normal liver tissues and cultured cell lines (human Hc, HuH-6, HuH-7, HepG2, rat RLC-16, and retinoblastoma cells (Y79)) indicated that the following characteristics were the unique points of the immortal cell lines; *i.e.*, [1] increase of +ssRNA virus , [2] occurrence of biotin production (adult humans could not synthesize biotin) (vitamin H) (6, 31, 32), [3] increase of biotinidase activity (Kip; repulsion power against produced biotin) (6), [4] appearance of D-aspartic acid (though the HepG2 showed a relatively low concentration) (5), [5] decreased ratio of membrane proteins (in the follows of this text), [6] increased production of histone H3.1 (3, 4). High concentration of biotin (both the total- and free-form) (32, 33) and high biotinidase activity (unpublished observation) was found in the chicken egg yolk. Interestingly, fucoidan seemed not to influence cellular gene expression in HepG2 at all; *i.e.*, histone content of non-treated and treated with fucoidan HepG2 cells were unexpectedly similar.

Apoptotic mechanism by Wakame-fucoidan (containing 11% of uronic acid) from "Wakame" (*Undaria pinnatifida*) has been recently reported by using the human HCC cell line SMMC-7721 (the Cell Bank of the Chinese Academy of Sciences, Beijing, China) (34), however stopping of cell division at the S-phase (DNA-replication phase) by fucoidan is not significant. Furthermore, apoptosis-related proteins such as caspases and the gene products of *Livin* (a member of the inhibitors of apoptosis protein family) and XIAP could not be detected in HepG2 and Hc, and liver biopsies using the DMD method. The increased biotinidase *K*ip in the HCC tissue was inhibited only by the sulfated glycochains of fucoidan and heparin with the exception of chondroitin sulfate (6), indicating that both the sulfation and component sugar of glycochain was important to inhibit the glycoprotein enzyme biotinidase. Fucoidan and heparin were also known to inhibit blood coagulation *via* binding to the glycochains of glycoprotein antithrombin III (35). Furthermore, anti-tumor and anti-metastatic activities of lung adenocarcinoma in mice exerted by the fucoidan from *Fucus evanescens* have already been reported (36). In this case, intra-peritoneal injection method of 10 mg/kg/day of

Table VIII. *Proteins of HepG2 cells without addition of fucoidan*.*

Proteins (Cellular proteins; 93.4%)	Amount (μg/mg of protein homogenate)
Neural cell adhesion molecule (841-)	3.3
WD repeat protein 72 (644-)	3.3
Hypoxia up-regulated 1 (15-)	*3.3*
Peroxisomal bifunctional enzyme (289-)	3.1
RING finger protein 123 (854-)	*3.0*
Gamma-Taxilin (118-)	2.9
Zinc finger protein 614 (174-)	2.9
SWI/SNF complex 155 kDa subunit (801-)	2.9
Uncharacterized protein C3 orf 25 (177-)	2.9
Neurogenin 2 (64-)	*2.8*
S-Arrestin (34-)	2.7
Dopamine D4 receptor (107-)	2.6
Sphingosine-1-phosphate phosphatase 1 (80-)	2.6
Hsp90 co-chaperone Cdc37 (13-)	2.5
Importin-7 (680-)	2.5
5'-3' Exoribonuclease 2 (180-)	2.5
Nucleotide-binding site protein 1 (310-)	*2.5*
Suppressor of tumorigenicity protein 14 (100-)	2.5
TGF-III receptor type III (84-)	*2.5*
DNA polymerase alpha catalytic subunit (1130-)	2.4
Excitatory amino acid transporter 5 (224-)	2.4
REST corepressor 3 (155-)	2.4
Transcription elongation factor SPT5 (355-)	*2.4*
L-Lactate dehydrogenase A-like 6A (12-)	2.3
Micrifibril-associated glycoprotein 3 (55-)	2.2
Poly (ADP-ribose) glycohydrolase (304-)	2.2
GPI mannosyltransferase 3 (251-) (Anchoring)	***2.2***
Metallocarboxypeptidase D (711-)	2.2
Zinc finger protein 366 (107-)	*2.1*
Isopentenyl-diphosphate delta-isomerase 2 (15-)	2.0
PDZ domain-containing protein 2 (2558-)	2.0
Uromodulin (13-)	*2.0*
Axin-1 (587-)	2.0
100 kDa DNA-pairing protein (431-)	2.0
Putative Rho-GTPase-activating protein FLJ46335 (281-)	*1.9*
Ankyrin repeat and fibronectin type-III domain-containing protein 1 (201-)	*1.8*
Zinc finger protein 710 (407-)	1.8
Na-Cl symporter (508-)	*1.7*
Vigilin (HDL-binding protein; 731-)	*1.7*
CREB-binding protein (2212-)	1.7
Tankyrase-1 (1088-)	1.7
RNA polymerase II subunit 5-mediating protein 288-)	1.6
Zinc finger protein 694 (469-)	*1.6*
Exportin-7 (111-)	*1.5*
Bestrophin-1 (379-)	1.5
F-box only protein 46 (198-)	*1.5*
HIV Tat-specific factor 1 (677-)	1.5
Neuroblastoma-derived sulphhydryl oxidase (251-)	1.5
Tenascin (Glioma-associated-extracellular matrix antigen; 1749-)	1.5
60S ribosomal protein L3-like (215-)	1.4
Biotin synthetase (Bacillus subtilis; 1-)	*1.3*
Gamma-aminobutyric-acid GABA(A)) receptor subunit gamma-2 (62-)	*1.3*
Gamma-Butyrobetaine dioxygenase (201-)	1.3
Leukocyte sialoglycoprotein (259-)	1.3
Transmembrane protease, serine 5 (46-)	1.3
Zinc finger protein 16 (493-)	1.3
ATP-dependent helicase 1 (643-)	*1.3*
Hypoxia-inducible factor 1alpha (646-)	1.3
Nucleoplasmin-2 (145-)	1.3
Opsin-3 (14-)	1.3
Squalene synthetase (40-) (Anchoring)	*1.2*
CD5 antigen-like (1-)	*1.1*
Cat eye syndrome critical region protein 5 (82-)	*1.1*
GRB2-associated-binding protein 1 (352-)	*1.1*
X-linked interleukin-1 receptor accessory protein-like 1 (547-)	1.1
Chordin-like protein 1 (305-)	1.0
High-affinity cAMP-specific 3',5'-cyclic phosphodiesterase 7A (188-)	1.0

*Proteins coming from membrane fractions were indicated in italics. Glycochain-synthesizing enzyme is indicated in bold.

fucoidan were used (36). Therefore, fucoidan may inhibit the above mentioned characteristics from [1] to [5] in immortalized cancer cells possibly through glycol-chains; *i.e.* [3] decrease of *K*ip of glycoprotein enzyme of biotinidase, [1] decrease of viral glycoproteins, and [5] the increase of the membrane compartment rich in glycoproteins, although the putative human genes and information of glycochain for [4] aspartate racemase and for [2] biotin synthetase are not yet available in the protein database (Protein BLAST in NCBI).

Attempts of growth-retardation tests directly by decreasing the intracellular concentrations of D-biotin and D-aspartic acid have not yet been successful. Thus, avidin addition in the culture medium was tested in order to reduce the intracellular biotin concentration, but the HeLa cells were not affected at all, since avidin-biotin complex (MW65,000) was actively taken-up by the cells (37). Since oral administration of the high-molecular-mass Okinawa-fucoidan attenuated DEN-induced liver fibrosis in Wistar rats (38), the liver cells seemed to be able to take-up the high-molecular-mass fucoidan (MW 200,000) *via* the gut. Furthermore, intraperitoneal injection of the high-molecular-mass fucoidan attenuated lung cancer in *C57Bl/6* mice (36), and the lung cells also seemed to able to take-up fucoidan from different regions of the body. Then, a direct supply of amounts of biotinidase to promptly growing fetus and baby was

Table IX. *Proteins of HepG2 cells without addition of fucoidan*.*

Proteins (Cellular proteins; 93.4%)	Amount (μg/mg of protein homogenate)
Reproduction 8 protein (135-)	1.0
Serine/threonine-protein kinase Nek5 (578-)	1.0
Breast tumor novel factor 1 (303-)	0.9
Odorant receptor HOR5'betall (27-)	*0.8*
Brain-specific serine protease 4 (56-)	0.8
Cold-inducible RNA-binding protein (60-)	0.8
Muscleblind-like X-linked protein (90-)	*0.8*
Protocadherin-17 (900-)	*0.8*
Olfactory receptor 4M1 (61-)	*0.8*
SH2 domain-containing protein 4A (336-)	0.8
Ankyrin repeat domain-containing protein 15 (1111-)	*0.8*
ATP-dependent helicase 1 (920-)	0.8
Discs large homolog 4 (479-)	*0.8*
Metalloproteinase inhibitor 4 (122-)	0.8
Methyltransferase-like protein 6 (150-)	0.8
Short stature homeobox protein 2 (229-)	0.8
Sperm equatorial segment protein 1 (113-)	*0.8*
Clusterin-associated protein 1 (376-)	0.7
Pinin (502-)	0.7
Zinc finger Ran-binding domain-containing protein 1 (607-)	0.7
Calmodulin-like protein 5 (57-)	*0.6*
Myosin-9B (1963-)	*0.6*
Titin immunoglobulin domain protein (302-)	*0.6*
Centromere/kinetochore protein zw10 homolog (585-)	*0. 6*
Ankyrin-2 (Brain ankyrin; 3735-)	*0.6*
Protein FAM11B (161-)	*0.6*
Alcohol dehydrogenase 6 (288-)	0.5
Desmuslin (Synemin; 1490-)	*0.5*
Olfactory receptor 52B6 (150-)	*0.5*
DNA-directed RNA polymerase III subunit G (176-)	0.5
mTERF domain-containing protein 2 (354-)	0.5
Aminopeptidase PILS (877-)	0.5
Insulin receptor substrate 2 (IRS-2; 1187-)	*0.5*
Glucose-6-phosphatase (G6Pase; 212-)	*0.5*
Claudin-4 (77-)	*0.4*
WW domain-containing oxidoreductase (283-)	*0.4*
Copine-2 (493-)	0.4
Histone-binding protein RBBP7 (Retinoblastoma-binding protein 7; 374-)	0.4
Mitochondrial 28S ribosomal protein S25 (59-)	*0.4*
SET and MYND domain-containing protein 3 (317-)	*0.4*
U4/U6,U5 tri-snRNP-associated protein 2 (445-)	*0.4*
Gamma-Adaptin-related protein 1 (536-)	*0.3*
Tumor suppressor candidate 2 (13-)	*0.3*
Uromodulin-like 1 (Olfactorin; 1269-)	*0.3*
RNA-binding protein TIA-1 (289-)	*0.3*
Lupus La protein (366-)	0.3
Integrin beta-8 (684-)	*0.3*
Taste receptor type 2 member 42 (236-)	*0.3*
Plasma membrane calcium ATPase isoform 3 (1145-)	*0.2*
Transcription factor Dp-1 (341-)	*0.2*
Syntaxin-12 (211-)	*0.2*
Transforming growth factor alpha (TGF-alpha; 97-)	*0.2*
Amidophosphoribosyltransferase (456-)	*0.2*
Whirlin (Autosomal redessive deafness type 31 protein; 848-)	*0.2*
Alpha-Albumin (Alpha-Alb; 573-)	0.2
Aw-80 (A-1; 310-)	*0.2*
Sodium-dependent multivitamin transporter (609-)	0.2
Visinin-like protein 1 (133-)	*0.2*
B-Lymphocyte activator macrophage expressed (230-)	*0.2*
Retinal pigment epithelium-specific 65 kDa protein (482-)	*0.2*
Tumor protein p53-inducible nuclear protein 1 (218-)	0.2
Doublecortin domain-containing protein 1 (259-)	*0.2*
GABA(A) receptor subunit pi (Internal 14mer; 418-431)	*0.1*
Glutathione peroxidase 1 (GSH Px-1; 162-)	*0.1*
Glutathione peroxidase 6 (183-)	*0.1*
XPA-binding protein 1 (337-)	*0.1*
Leptin receptor overlapping transcript-like 1 (105-)	*0.1*
UPF 0258 (84-)	0.1
Glycophorin E (21mer; 58-)	*0.1*
Glutathione synthetase (458-)	*0.1*
Biotinidase (1-; fetal-type sequence of Cole *et al*. [13])	0.0
Total	934

*Proteins coming from membrane fractions are indicated in Italics.

suggested, since chicken egg yolk (unpublished observation) and human breast milk (39) contained relatively high biotinidase. Recently, the direct demonstration of dependence on biotin for the growth of the mutant bacterium *Mycobacterium tuberculosis* has been reported (40). Our testing of addition D-aspartic acid (10 mM) in the medium of RLC-16 (immortalised epitherial cell line established in Japan from the liver of *Rattus norvegicus*), which required L-glutamine (10 mM) in the medium, had also been unsuccessful (5); *i.e.*, extremely high concentrations of D-aspartic acid (product) might have inhibited the aspartate racemase reaction. Then, reduction of intracellular biotin and D-aspartate might have been achievable by addition of mercury ion, which is a common inhibitor to biotin synthetase (41) and to aspartate racemase (42). Therefore,

Table X. *Proteins of HepG2 cells treated with fucoidan for 3 days*.*

Proteins (Cellular proteins; 95.4%)	Amount (μg/mg of protein homogenate)
Titin (9821-)	*65.4*
Mucin-16 (Ovarian cancer related tumor marker CA125; 9643-)	64.8
Titin (9391-)	63.8
Titin (18190-)	41.8
Apolipoprotein B-100 (Apo B-100; 359-)	21.8
Ceruloplasmin (from 214- of pre)	20.5
Ceruloplasmin precursor (from 1-)	*16.5*
Flamingo homolog 2 (73-)	15.1
Probable ATP-dependent RNA helicase DHX37 (295-)	13.3
Histone H 3.1. (2-)	12.9
Transferrin precursor (TFpre; 1-)	12.7
Insulin-like growth factor II receptor (IGF-II receptor; 199-)	11.8
Laminin subunit alpha-2 (765-)	11.7
Deubiquitinating enzyme 19 (526-)	*10.8*
DNA polymetase kappa (28-)	*10.8*
Stabilin-1 (454-)	10.5
Serum (sBIN) biotinidase (1-)	9.9
Ether-a-go-go potassium channel 1 (365-)	9.8
Alpha-N-Acetylglucosaminidase (NAG; 1-)	*9.5*
A-kinase anchor protein 9 (452-)	*9.4*
Ceruloplasmin (from 19- of pre)	9.4
Apolipoprotein B-100 (Apo B-100; 2723-)	9.2
Semaphorin-6c (930-)	9.1
Endothelin-converting enzyme-like 1 (84-)	*8.9*
Molecule interacting with CasL protein 1 (209-)	8.9
Leucine-zipper-like transcriptional regulator 1 (138-)	*8.8*
Protein KIAA 0423 (92-)	8.3
Protein KIAA 1199 (722-)	*8.0*
Squamous cell carcinoma antigen recognized by T cells 1 (123-)	7.0
DNA mismatch repair protein Mlh3 (42-)	7.0
Zinc finger protein 518 (154-)	6.6
G2/mitotic-specific cyclin-B3 (201-)	6.2
Protein KIAA 0195 (208-)	6.1
Collagen alpha-1 (XVIII) chain (378-)	5.9
Tubulin-folding cofactor D (43-)	5.9
Lipoamidase precursor (mLIP; CEL; BSSL; 1-)	5.8
Signal transducer and activator of transcription 4 (183-)	5.8
Albumin precursor (from 1-)	5.7
Down syndrome cell adhesion molecule (1-)	*5.7*
Homeodomain-interacting protein kinase 2 (105-)	5.7
Probable G-protein coupled receptor 112 (2358-)	*5.7*
Lipoamidase (May be LIP/BIN; Enterococcus faecalis; 1-)	5.6
Mucin-6 (Ovarian carcinoma antigen CA125; 11371-)	5.5
Myosin-15 (1381-)	*5.5*
Neuroligin-1 (425-)	*5.3*
Cancer-amplified transcriptional coactivator ASC-2 (116-)	5.2
Melanocyte protein Pmel 17 (160-)	5.2
Raptor (314-)	*5.2*
Vigirin (228-)	5.2
Apolipoprotein-L1 (1-)	*5.1*
Neuroendocrine sodium channel (1578-)	*5.1*
Biotin synthetase (Bacillus subtilis; 1-)	*5.1*
BRG1-associated factor 170 (202-)	5.0
Urine (kidney-type; uBIN) biotinidase (1-)	*5.0*
Maltase-glucoamylase, intestinal (12-)	*4.8*
Histone H 2A (2-)	4.7
Semaphorin-6A (127-)	4.7
Histone H 2B (2-)	4.6
G/T mismatch-binding protein (436-)	4.6
Transmembrane protein 9B (18-)	*4.6*
Integrin alpha-2 (345-)	4.3
Zinc finger MYM-type protein 1 (340-)	*4.3*
Sodium/bile acid cotransporter (23-)	*4.2*
Baculoviral IAP repeat-containing protein 6 (3310-)	*4.2*
HIV-1 Nef-interacting protein (T-complex protein 1 subunit eta; Mr 16000; 401-)	***4.2***
LDL receptor (24-)	4.2
Inositol-triphosphate 3-kinase C (366-)	*4.1*
alpha-Fetoprotein (from 1-)	4.0
Dedicator of cytokinesis protein 9 (1314-)	3.9
Lipoic acid synthetase (Human; 1-)	3.9
Exportin-T (588-)	3.9

*Fucoidan from Ishi-Modzuku was added at 0.102 mg/mL weight/ml DMEM for 3 days. Proteins coming to the membrane fractions are indicated in Italics. Titin is a serum marker of adulthood; *i.e.* titin is detectable in HepG2 (from male HCC liver of 15 years old patient) but not detected in Hc (from fetal livers).

the growth of the HCC-derived cultured cells may be expected to be slowed-down by the edible fucoidan through the above suggested five signals.

Although data obtained from hepatocarcinoma, such as HuH-7 cells, might be necessary to draw the conclusion, fucoidan from edible Ishi-Mozuku also seemed to change the amounts of cellular proteins; *i.e.* (1) increase of ceruloplasmin (1.4-fold), transferrin (1.6-fold), glycol-chain synthesizing enzymes (2.2-fold) and membrane receptors (1.4-fold) (Tables VII to XIII), and (2) decreases of non-liver proteins of albumin (3.3-fold; skeletal muscle), alpha-fetoprotein (AFP; 1.9-fold; fetal liver), myosin (1.4-fold; muscle), titin (1.2-fold; muscle), protocadherin (brain) 8.1-fold, hypoxia-inducible factor (brain) 3.5-fold, and lipoamidase (1.8-fold; brain) (Tables VII to XIII). Increases in ceruloplasmin and transferrin

Table XI. *Component proteins of HepG2 cells treated with fucoidan for 3 days*.*

Proteins (Cellular proteins; 95.4%)	Amount (μg/mg of protein homogenate)
ATP-binding cassette subfamily A member 6 (186-)	*3.8*
Collagen alpha-1 (XVIII) chain (103-)	*3.8*
Histone H 4 (2-)	3.7
Ran-binding protein 6 (360-)	3.7
Uncharacterized protein C6 orf 167 (527-)	3.7
ADAMTS-like protein 2 (250-)	3.6
Semaphorin-5B (383-)	3.6
Aggrecan core protein (1720-)	3.6
Microtubule-associated protein 2 (MAP 2; 543-)	*3.5*
Choline transporter-like protein 2 (43-)	*3.4*
3-Hydroxyacyl-CoA dehydrogenase (91-)	3.3
Friend of EBNA2 protein (14-)	*3.1*
Lipoic acid synthetase (Bacillus subtilis; 1-)	3.1
Gelatinase B (161-)	2.8
Glycogen [starch] synthase, muscle (174-)	2.8
5-Azacytidine-induced protein 1 (530-)	2.8
DNA polymerase alpha catalytic subunit (418-)	*2.8*
Nesca (362-)	2.8
SPS1/STE20-related protein kinase YSK4 (780-)	2.7
HeLa tumor suppression 1 (121-)	*2.7*
Ankyrin repeat domain-containing protein 38 (15-)	*2.6*
Protein cordon-bleu (285-)	*2.5*
DNA polymerase theta (1266-)	2.5
T-lymphoma invation and metastasis-inducing protein 1 (648-)	*2.4*
Probable JmjC domain-containing histone demethylation protein 2C (1624-)	*2.4*
Vanilloid receptor-like protein 1 (578-)	*2.4*
C-myc promoter-binding protein (975-)	*2.3*
Excitatory amino acid receptor 5 (830-)	*2.3*
ATP-binding cassette 12 (1808-)	*2.2*
Chromobox protein homolog 2 (136-)	2.0
ADAMTS-4 (137-)	*1.9*
Somatostatin receptor type 1 (SS1 R; 267-)	1.9
Hepatocyte growth factor-regulated tyrosine kinase substrate (81-)	*1.9*
Lipoamidase precursor (mLIP; CEL; BSSL; 4-)	*1.9*
Chromosomal protein of 68 kDa (67-)	*1.8*
Ankyrin repear and fibronectin type-III domain-containing protein 1 (108-)	*1.8*
Myosin-1 (1584-)	1.8
Probable G-protein coupled receptor 111 (53-)	*1.8*
Hyaluronan synthase 3 (215-)	**1.7**
WWC family member 3 (363-)	*1.7*
Alpha-(1,3)-Fucosyltransferase (18-)	***1.7***
Inner membrane protein Man1 (265-)	*1.7*
Zinc finger SWIM domain-containing protein 5 (856-)	1.7
26S Protease regulatory subunit 6B (95-)	1.6
alpha-Fetoprotein precursor (from 1-)	*1.6*
Ankyrin-2 (Brain ankyrin; 3335-)	*1.6*
Attractin (1109-)	1.6
Integrin alpha-IIb (474-)	*1.6*
25-Hydroxyvitamin D-1 alpha hydroxylase, mitochondrial (212-)	1.5
Ankyrin-3 (3784-)	*1.5*
Cadherin-18 (513-)	*1.5*
Delayed-rectifier K(+) channel alpha subunit 3 (202-)	1.5
Deubiquitinating enzyme 29 (633-)	1.5
cGMP-dependent protein kinase 2 (224-)	*1.4*
Human immunodeficiency virus type I enhancer-binding protein 2 (HIV-EP2; 1917-)	*1.4*
Rab proteins geranylgeranyltransferase component A 2 (109-)	*1.4*
Acyl-coenzyme A oxidase 2, peroxisomal (169-)	*1.4*
Aftiphilin (428-)	*1.4*
Angiopoietin-4 (14-)	*1.4*
BTB/POZ domain-containing protein 12 (874-)	1.4
Chloride intracellular channel 6 (172-)	*1.4*
G patch domain-containing protein 2 (17-)	*1.4*
Matrix metalloproteinase-19 (244-)	1.4
Pumilio homolog 1 (643-)	*1.4*
Arginyl-tRNA transferase 1 (37-)	*1.3*
ATP-binding cassette 12 (2106-)	*1.3*
Phospholipid scramblase 1 (50-)	*1.3*
Cadherin-10 (527-)	1.3
Probable helicase senataxin (2220-)	*1.3*
Protocadherin-17 (695-)	*1.3*
Neprilysin (283-)	*1.2*
Pre-mRNA cleavage complex 2 protein Pcf11 (1411-)	1.2

*Fucoidan from Ishi-Modzuku was added at 0.102 mg dry-weight/ml DMEM medium for 3 days. Proteins coming from the membrane fractions are indicated in Italics. Glycochain-synthesizing enzymes were indicated in bold.

turned-out to be non-general; *i.e.*, a liver of pseudo-cancer showed similar ceruloplamin levels to HCC tissues, and LC tissue of a liver of an HCC patient showed a decreased transferrin amount compared to HCC tissue. Decrease of albumin and AFP might also not be general; *i.e.*, although one HCC patient showed a decrease of albumin in HCC using chromato-graphic analysis (4), but increased albumin in HCC tissue was observed in an HCC patient, and increased AFP in the LC tissue of the leprocy patient was also observed using the microsequencing method. Instead, changes in the glycochain of AFP seemed more important; *i.e.*, the highly-enhanced fucosylation of AFP in patients with germ cell tumor has already been reported (43). Since protein analysis of the liver tissues had been performed under the still unimproved micro-sequencing method, changes in the minor proteins seem to be meaningless.

Table XII. *Component proteins of HepG2 cells treated with fucoidan for 3 days*.*

Proteins (Cellular proteins; 95.4%)	Amount (μg/mg of protein homogenate)
Serine protease TADG-15 (407-)	*1.2*
Trap 222 (1334-)	1.2
Excitatory amino-acid carrier 1 (95-)	*1.2*
Glucocorticoid receptor (GR; 549-)	1.2
Inositol hexaphosphate kinase 3 (190-)	1.2
Melanoma-associated antigen D4 (304-)	*1.2*
Chloride intracellular channel 6 (262-)	*1.1*
MICAL-like protein 2 (485-)	*1.1*
TBC1 domain family member 20 (9-)	*1.1*
Uncharacterised protein C14 orf 54 (202-)	*1.1*
Heterogeneous nuclear ribonucleoprotein M (hnRNP M; 338-)	*1.1*
Iroquois-class homeodomain protein IRX-4 (304-)	*1.1*
Oral-facial-digital syndrome 1 protein (806-)	*1.1*
Probable G-protein coupled receptor 126 (832-)	*1.1*
Sorting nexin-6 (2-)	*1.1*
Tumor necrosis factor type 1 receptor-associated protein 2 (510-)	*1.1*
GABA(A) receptor subunit delta (82-)	*1.0*
HepA-related protein (583-)	*1.0*
Monocyte differentiation antigen CD14 (238-)	*1.0*
Outer mitochondrial membrane protein porin 2 (154-)	*1.0*
Peroxisomal membrane protein PMP34 (19-)	*1.0*
Lymphoid blast crisis oncogene (95-)	*0.9*
Platelet-activating factor acetylhydrolase IB subunit alpha (227-)	0.9
Acetylcholine receptor protein subunit delta (197-)	*0.9*
EGF-like module-containing mucin-like hormone receptor-like 2 (655-)	0.9
Zinc finger protein 445 (715-)	*0.9*
Guanine nucleotide-binding protein-like 1 (142-)	*0.8*
Inositol-triphosphate 3-kinase C (367-)	*0.8*
Leucine-rich repeat-containing G-protein coupled receptor 6 (665-)	*0.8*
Receptor activity-modifying protein 2 (20-)	0.8
Sialidase-4 (327-)	0.8
Trace amine-associated receptor 2 (52-)	*0.8*
Ester hydrolase C 11 orf 54 (24-)	*0.8*
Leucine-rich repeat transmembrane protein FLRT1 (356-)	*0.8*
Zinc finger protein 460 (286-)	0.8
Acyl-CoA wax alcohol acyltransferase 1 (186-)	0.7
Histone H3-K9 methyltransferase (457-)	*0.7*
Polycystic kidney disease 2-like 2 protein (358-)	*0.7*
Protocadherin alpha 13 (679-)	0.7
Sialic acid-binding Ig-like lectin 8 (247-)	*0.7*
Ubiquinol-cytochrome c reductase iron-sulphur subunit, mitochondrial (143-)	0.7
Chollagen alpha-3 (IX) chain (550-)	*0.7*
DAZ-associated protein 1 (172-)	*0.7*
Hypoxia-inducible factor 1 alpha (594-)	*0.7*
Protocadherin alpha 5 (677-)	0.7
Ran-binding protein 3 (540-)	*0.7*
Zinc finger CCCH-type domain-containing protein 7A (852-)	0.7
Alpha-2,8-Sialyltransferase 8E (152-)	***0.6***
Beta-1 Adrenergic receptor (431-)	*0.6*
Hyaluronidase-3 (299-)	0.6
Integral membrane protein 2B (40-)	*0.6*
Protein Mpv 17 (54-)	0.6
Protein transport protein Sec 61 (243-)	*0.6*
Stromal membrane-associated protein 1 (237-)	0.6
Transmembrane protein 22 (197-)	0.6
Calcium/calmodulin-dependent protein kinase type 1D (270-)	0.6
Hypoxia up-regulated 1 (779-)	*0.6*
Leukemia-associated protein 5 (297-)	0.6
Na(+)/glucose cotransporter 3 (447-)	*0.6*
Probable palmitoyltransferase ZDHHC19 (66-)	*0.6*
Probable phospholipid-transporting ATPase IIA (836-)	*0.6*
UDP-GlcA/UDP-GalNAc transporter (159-)	*0.6*
UDP-Glucuronosyltransferase 2B11 (321-)	*0.6*
RAD51-associated protein 1 (159-)	*0.5*
Kidney and brain protein (1016-)	0.5
Biotin synthetase (Helicobacter pylori J99; 1-)	0.5
Endoribonuclease Dicer (1728-)	*0.5*
Iodotyrosine dehalogenase 1 (113-)	*0.5*
Phosphatase and actin regulator 2 (458-)	*0.5*
Coiled-coil domain-containing protein 53 (24-)	*0.5*
GTP-binding protein 3 (400-)	*0.4*
Diacylglycerol kinase theta (DAG kinase theta; 859-)	0.4
Dermatan sulphate proteoglycan 3 (159-)	*0.4*

*Fucoidan from Ishi-Modzuku was added at 0.102 mg dry-weight/mL DMEM medium for 3 days. Proteins coming from the membrane fractions were indicated in Italics. Glycochain-synthesizing enzymes were indicated in bold.

Thus, only the ubiquitously-expressed protein of lipoamidase, glycol-chain synthesizing enzymes, and membrane receptors might have certain meaning as described below. The above discussed results of changes in cellular protein amounts are in line with those of previous results using 2-dimensional electrophoresis analyses regarding sexual differentiation (8).

On the other hand, fucoidan seems not to change the amounts of biotinidase, histone, ankyrin, and membrane active-transport proteins at all (Tables VII-IX and X-XIII).

Membrane active-transport proteins are constitutively important proteins for cell viability through nutrition uptake (44) and against toxins (mercuric ion) (45). Histone is not detected in liver of pseudo-cancer as expected, but is highly

Table XIII. *Proteins of HepG2 cells treated with fucoidan for 3 days*.*

Proteins (Cellular proteins; 95.4%)	Amount (μg/mg of protein homogenate)
Long-chain farry acid transport protein 4 (561-)	0.4
Gastric intrinsic factor (336-)	*0.4*
Arginine/serine-rich-splicing factor 14 (929-)	*0.4*
Multiple epidermal growth factor-like domains 9 (524-)	0.4
Vacuolar ATP synthase subunit D (97-)	*0.4*
Serine protease inhibitor Kazar-type 4 (8-)	0.4
Dihydrofolate reductase-like protein 1 (111-)	0.4
Alzheimer disease amyloid protein (699-)	0.4
Sensory neuron sodium channel 2 (1658-)	*0.4*
Red-sensitive opsin (294-)	0.3
Fibroblast growth factor 23 (125-)	*0.3*
Arginyl-tRNA--protein transferase 1 (402-)	*0.3*
Cysteine-rich secretory protein 1 (135-)	*0.3*
Secretory carrier-associated membrane protein 1 (220-)	*0.3*
Taste receptor type 2 member 42 (251-)	0.3
BTB-like protein (419-)	*0.3*
Serine/threonine-protein kinase Krs-1 (434-)	*0.3*
Transcription factor HES-4 (165-)	0.3
Zinc finger and SCAN domain-containing protein 2 (507-)	*0.3*
1,4-alpha-Glucan branching enyme (594-)	***0.3***
Cell division control protein 2 homolog (187-)	0.3
Macropain subunit C5 (138-)	*0.3*
Tyrosinase (476-)	0.3
Adenylyl cyclase-associated protein 1 (381-)	*0.3*
Uncharacterized protein C 20 orf 24 (37-)	*0.3*
Keratinocyte growth factor (98-)	*0.2*
WD repeat protein 89 (303-)	*0.2*
Lanp (164-)	*0.2*
System N amino acid transporter 1 (423-)	*0.2*
Tumorigenic protein pp32rl (160-)	*0.2*
Ribonuclease-like protein 10 (182-)	0.2
Group VI phospholipase A2 (773-)	0.2
Poliovirus receptor-related protein 1 (172-)	*0.2*
Plexin-B2 (1789-)	*0.1*
Teashirt homolog 1 (1027-)	*0.1*
Smoothelin (878-)	*0.1*
Sushi domain-containing protein 2 (785-)	*0.1*
Dipeptidyl-peptidase 1 (429-)	*0.1*
Histone deacetylase 7a (HD7a; 916-)	*0.1*
Interleukin-21 (IL-21; 121-)	*0.1*
Protein S100-B (58-)	*0.1*
Arginine decarboxylase (427-)	*0.1*
Cathepsin F (453-)	0.1
Lymphocyte antigen Ly-6D (113-)	*0.1*
Exportin-5 (17mer; 1188-)	*0.04*
Induced myeloid leukemia cell differentiation protein Mcl-1 (17mer; 334-)	*0.04*
Intraflagellar transport 88 homolog (17mer; 808-)	*0.04*
Uncharacterized protein C 13 orf 16 (135-)	*0.04*
T-cell leukemia homeobox protein 2 (15mer; 270-)	*0.04*
Transcription factor HMX3 (12mer; 1119-)	*0.04*
Polymerase delta-interacting protein 2 (11mer; 358-)	*0.03*
Biotinidase (1-; fetal-type sequence of Cole *et al*. [13])	0.00
Total	955

*Fucoidan from Ishi-Modzuku was added at 0.102 mg dry-weight/mL DMEM medium for 3 days. Proteins of the membrane fractions are indicated in italics. Glycochain-synthesizing enzyme was indicated in bold.

expressed (1.2%) in the LC tissue of leprosy patients. Histone may not be important for the cancer, but may be essential for the immortality of cells. Ankyrin (an S-palmitoylated anchored membrane protein) is also essential for cell viability through maintaining the cell morphology (46), but does not appear in the liver tissues except LC and HCC tissues of one HCC patient with *HCV*. Therefore, biotinidase and membrane active-transport proteins seem to be essentially important for cell viability. Fucoidan does not influence gene expression of these essential proteins regarding viability of the cells; *i.e*., fucoidan may not act on the cell death mechanism of the cancer cells such as apoptosis. Then, this is the reason why fucoidan is a safe drug without side-effects on normal cells and organs.

An increase of biotinidase activity (*K*ip; inhibition constant by the product biotin, and Cap) in HCC tissues has been recently found (6). However, the amount of biotinidase protein in HepG2 cells (from an HCC patient of 15 years) is not changed (from 16.4 to 14.9 μg/mg) by treatment with fucoidan (Tables VII-IX and X-XIII). Interestingly, the amount of serum biotinidase in the biotin-deficient patient is also not changed by administration of biotin (10 mg/day) (19). These findings suggest that biotinidase is the non-inducible (constitutively expressed) protein indispensable for growth and viability of adult humans.

The fetal liver biotinidase (Hc cells; 6.2 μg/mg) is firstly found at this time (Table IV), which suggests the important role of fetal biotinidase during fetal growth. Human serum biotinidase seems to be expressed in an age-depended differentiation manner; *i.e*., gradual increase of serum biotinidase to the adult level within 3 months after parturition, and the age-dependent gradual increase (highest activity; at 18.7 years of age), and decrease of the activity level to zero at the age of 174 years (47). Biotinidase is a known glycoprotein enzyme (48), and ubiquitous biotinidase activity in rat tissues are variable according to tissue

differences; *i.e.*, tissue difference of biotinidase activity occurs not by difference in biotinidase gene but the different glycochain of biotinidase (12, 47, 48). Then, decrease of biotinidase activity together with decrease of biotin by fucoidan may be caused by a possible binding of fucoidan to the glycochains of biotinidase.

Membrane-glycoprotein-enzyme lipoamidase is rich in the pig brain membrane (49) although not yet determined in the human brain, and lipoamidase was found to be decreased by fucoidan in HepG2 cells. Lipoamidase has been known to catalyze the de-anchoring reaction for N-myristoylated and glycosyl-phosphatidyl-inositol (GPI)-bonded membrane-bound enzymes (50). Lipoamidase is also thought to be the recycling enzyme of lipoic acid (49, 50). Therefore, lipoamidase activity (47) and lipoic acid concentration (16) may be correlated. Then, the decrease of de-anchoring amidase (tissue-type lipoamidase) protein also seems to occur through changes in glycol-chains of the enzyme biotinidase. Although lipoamidase could hydrolyze the *S*-palmitoyl-anchored membrane proteins or not is as yet not established (50), S-palmitoylated viral proteins of genome polyprotein (*HCV*) and envelope glycoprotein gp160 (*HIV*) in HepG2 cells seem to be de-anchored (Table V). The ratio of cellular membrane proteins to the total cellular proteins was found to be increased from 29.0% to 43.2% by fucoidan treatment in HepG2 cells after three days (membrane proteins are indicated in Italics in (Tables VII-IX and X-XIII). Thus, the immediate increase of the ratio of membrane by fucoidan may be related to the direct inhibition of glycoprotein enzyme of de-anchoring enzyme lipoamidase by fucoidan *via* its glycochain. A similar direct inhibition of glycoprotein enzyme of biotinidase by Okinawa-fucoidan was recently reported by our group (6). The ratio of membrane proteins to the total cellular proteins is usually approximately 50% in normal liver biopsy specimens; *i.e.*, pseudo-liver cancer of 48.2%, LC tissue of leprosy 52.5%, LC tissue 36.0% and HCC tissue 26.5% of *HCV* infected patient, and HCC tissue of PBC patient 13.6%, respectively. Interestingly, Hc cells (immortal but non-cancer cell) from fetal tissue show that cellular membrane proteins are 23.9% of the total cellular proteins. This suggests that the ratio of membrane proteins seems directly linked to immortality (Tables II, III, IV). Since the ratio of membrane proteins to the total cellular proteins can be precisely, easily, and promptly determined within 3 h; *i.e.*, tissue homogenate is ultra-centrifugated at 100,000 ×*g* for 1.5 h, and concentrations of proteins in the separated precipitated membrane- and soluble supernatant-fractions are determined by the fast SEC (3), thus, this ratio may become the most reliable diagnostic method for HCC and immortality. The incorporation of membrane proteins (or precursor membrane proteins) into the membrane structure requires for suitable metabolic conditions of glucose (51) and biotin concentration (52). Thus, the amount or ratio of membrane may be depended on the lipoamidase protein and/or lipoamidase activity regulated by glycolchain, and on the metabolic state of the cells. Therefore, immortalized cell lines and HCC tissues may be in the condition of "de-anchored" or "state of the decreased membrane-protein".

Fucoidan increases glycochain-synthesizing enzymes (from 2.2 to 4.9 μg/mg; 2.2-fold; Tables VII-IX and X-XIII in bold brown), although not yet conclusive since the database of genes of glycochain-synthesizing enzymes were uploaded only recently. However, this increase may be important for acquiring the normalized immunity through normalized glycolchains of the glycoproteins. Therefore, it is also interesting that fucoidan *per se* has the ability to perform innate immunity in the invertebrate shrimp (53). Since the reduction of +ssRNA virus, HCV and HIV are over 10-fold, the effect of fucoidan on the cellular protein synthesis linked to the differentiation (or gene expression) seems to be small or slow. Therefore, fucoidan may have a direct effect on glycol-chain synthesis and/or metabolism of the glycoproteins. Indirect therapies using glycol-chain compounds or immunological polysaccharides as antigens have already been performed on cancer patients in Japan; *i.e.*, glycochain antigen prepared from human-type tubercle bacilli SSM (Special Substance of Maruyama; *Maruyama vaccine*) (54) and glycol-lipid antigens prepared from the own or another patient's urines (*Hasumi vaccine*) (55). Because fucoidan seems to have a direct effect on glycobiology and immunology together with the reduction of HIV and HCV on HepG2, the experiment for the therapy of chemically induced HCC by edible fucoidan from Silky-Mozuku in the rat has already been performed by us (56). It has been found that the successful therapy is observed only in the *SD* rat (outbred; constitutively possessing the normal immunity), but the tests with the *LEW* rat (inbred; without constitutively possessing the immunity genetically; generally used in the transplantation experiment) are all in vain (56).

In addition to the effects on proteins and glycoproteins, fucoidan from Japanese edible Ishi-Mozuku (*Sphaerotrichia divaricate*) at 0.102 mg/mL surely retarded the growth of HepG2 cells at 3 day's treatment; collected and washed cell volume was decreased to 1/2 of the cell volume of untreated control, and two-fold lower volumes of homogenate were obtained, which was safely employed to comparative studies (Tables V to XIII). However, this fucoidan at 0.20 mg/mL did not retard the growth of normal Hc cells. On the other hand, it is noteworthy that fucoidan from Ireland's non-edible Mozuku (*Fucus vesiculosus*) at 0.20 mg/mL killed and detached the Hc cells immediately within 3 days, and Hc cells could not be harvested using the cell scraper. With respect to apoptosis (or programmed cell death), apoptosis-related proteins (deoxyribonuclease, caspases, products of the genes p53, Livin, and XIAP) were not detected in HepG2

cells (both with and without fucoidan), in Hc cells, and liver tissues (both HCC and LC tissues) at all. However, by MTT assay measuring the mitochondrial-reducing ability of living cells with deteriorated cell membrane is widely employed for detecting apoptosis in biotechnological studies. The trypan blue method, by using another dye, is also used to measure dead cells with disrupted cell membranes. Therefore, the cellular-toxicity test (or apoptosis test) using lower concentrations of Ireland's non-edible Mozuku (*Fucus vesiculosus*) on Hc cells by the MTT assay may still be remained to be performed together with the direct volume-based growth test.

Analysis of gene expression is important for biological and clinical researches; *i.e.*, differentiation, development, ageing, cancer, and diseases researches. Analysis of messenger RNA (mRNA) instead of proteins is an indirect method, and it uses a hybridization technique with expensive DNA probes. Two-dimensional (2D) electrophoresis seems to be a direct protein analytical method, however electrophoresis of hydrophobic membrane proteins and the very-large MW proteins, such as titin, still remains a difficult issue (57). Therefore, sexual differentiation in response to mating hormone (S-farnesyl-dodecapeptide) has been previously studied by using only the soluble hydrophilic cellular proteins by our group, which indicated that unexpectedly small differences in the gene expression have occurred (8). On the other hand, this micro-sequencing method analyzes directly and generally both the membrane and soluble proteins expressed in the cells including viral and bacterial invaded-microbe proteins.

Thus, this micro-sequencing method is proven to be powerful tool for the identification and determination of the component proteins of biological specimens, which are useful in biosciences, such as clinical chemistry, diagnosis and therapy in medicine, biology, biochemistry, and biotechnology.

Conclusion

Approximately 250 proteins (including membrane proteins and invaded-microbe and viral proteins) are detected and determined in liver cell lines by the direct microsequencing-deciphering method.

This method is a direct and quantitative method; *i.e.*, this method is depended solely on the fidelity and preciseness of the automated-Edman reaction and determination of the PTH-amino acid with RP-HPLC.

It is found that edible fucoidan dramatically increases the ratio of membrane proteins and reduces +ssRNA viruses such as HCV and HIV in HepG2 hepatocarcinoma cells.

The mechanism of immortality based upon the glycobiology of HepG2 cells is presented herein. Furthermore, the prevention mechanism of this immortal growth of HepG2 by sulphated-polyfucose (fucoidan) has also been presented by the glycobiological and nutritional point of view.

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