Antitumorigenic Effects of Several Food Proteins in a Rat Model with Colon Cancer and Their Reverse Correlation with Plasma Bile Acid Concentration

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Summary In order to obtain information on the preventive effects of various food proteins against colonic cancer, six groups of azoxymethane-initiated mature Fischer rats (n=10) were fed respective diets different in protein sources such as bovine milk casein (casein), high-molecular-weight fraction from protolytic digest of soy protein isolate (soybean HMF), hen’s yolk defatted protein (yolk protein), wheat gluten and codfish meat, which had been supplemented with sodium deoxycholate (hereinafter, DCA) as a cancer promoter except for an additional DCA-unfed casein group. All of the living rats at checkpoints during the feeding period were examined by the use of a bronchus fiberscope for colonic tumor incidence at 6 wk intervals between the 10th and 34th wk, from which both blood and feces samples were taken at times of endoscopy. Tumorigenesis in the colon was perceived by endoscopy at wk 22 in the group fed DCA casein only and at wk 28 in the other groups except the DCA-unfed casein group. At wk 34, both soybean HMF and yolk protein groups ranked inferior to the DCA-unfed group in tumor incidence. When plasma steroid or lipid concentration was plotted against tumor incidence at wk 28 or 34, positive correlations were found between plasma bile acid concentration and tumor incidence at both weeks. With the exception of the DCA-unfed casein group, plasma bile acid concentration was reversely correlated to fecal bile acid excretion. Taken altogether, these results suggest that bile acids at higher concentrations in the plasma may serve as risk factors of colon tumor incidence.

Key Words plasma bile acid, colonic cancer, endoscopy, tumor incidence, dietary deoxycholate

It is generally accepted that secondary bile acids arising from microflora-mediated dehydroxylation of primary bile acids serve as cancer-promotive factors in the colon. There is, however, little information about the mechanism of action by which secondary bile acids promote carcinogenesis in the colon. Alternatively, a lowering of the plasma cholesterol level is brought about by intrahepatic conversion of cholesterol to bile acids and their fecal excretion in large amounts. One of the representatives of hypcholesterolemic foodstuffs is soybean HMF, which is a sedimental “high-molecular-weight” fraction separable by centrifugation from the proteolytic digest of soy protein isolate with microbial enzymes (1–3). Soybean HMF intake causes high bile acid excretion into the feces and seems to be more effective in prevention against experimental tumorigenesis than milk casein intake (4, 5). This effect can be accounted for by the high capacity of HMF to absorb or mask bile acids in the intestine. Such nutritionally unavailable ingredients in HMF digest are regarded as a sort of dietary fiber which is referred to as “resistant protein.” Resistant proteins exist in cereal protein isolates as well, serving as regulators of intestinal estrogen (6), cholesterol absorption (7) and short-chain fatty acid fermentation (8). Their existence in other plant or animal protein isolates may well be inferred by analogy with previous observations. This investigation was designed to compare the preventive effect of HMF, strictly speaking “soybean resistant protein,” against colonic tumor development with those of several food proteins using a fiberscope and concomitantly inspecting the process of oncogenesis in aged rats relating to the promoter action of secondary bile acids (9–11).

MATERIALS AND METHODS

Animals and feeding. Rats called “retired ones” that have too grown to meet the purpose of nutritional evaluation are rather favorable for long-term tumorigenicity tests because of tolerant attention to body weight change. For this reason, mature male F-344 rats at least 300 g in weight were purchased from Shimidzu Laboratory Supplies Ltd., Kyoto. Protein sources chosen for the preparation of diets were soybean HMF, milk casein, yolk protein, wheat gluten and codfish meat, of which the contents in the diets were adjusted to 10% (cellulose 5%) on a dry weight basis except for 13.5% soybean HMF (cellulose 1.5%) because about a quarter...
Table 1. Composition of experimental diets (%).

| Ingredient                  | Milk casein | Soybean HMF | Wheat gluten | Yolk protein | Codfish meat | Casein (-DCA) |
|-----------------------------|-------------|-------------|--------------|--------------|--------------|---------------|
| Protein source              | 10.0        | 13.5        | 10.0         | 10.0         | 10.0         | 10.0          |
| α-Cornstarch                | 73.8        | 73.8        | 73.8         | 73.8         | 73.8         | 73.8          |
| Soybean oil                 | 5.0         | 5.0         | 5.0          | 5.0          | 5.0          | 5.0           |
| Mineral mixture             | 5.0         | 5.0         | 5.0          | 5.0          | 5.0          | 5.0           |
| Vitamin mixture             | 1.0         | 1.0         | 1.0          | 1.0          | 1.0          | 1.0           |
| Cellulose                   | 5.0         | 1.5         | 5.0          | 5.0          | 5.0          | 5.0           |
| Na deoxycholate             | 0.2         | 0.2         | 0.2          | 0.2          | 0.2          | 0.0           |

Abbreviations are as follows: HMF, an insoluble "high-molecular-weight" fraction from microbial protease-treated soy protein; casein (-DCA), milk casein without supplemental deoxycholate. Soybean HMF and soybean oil were supplied from Fuji Oil Co., Osaka. Yolk protein (81.4% in protein) was a gift of Taiyo Kagaku Co., Mie. Codfish meat (>85% in protein) was presented from Nihon Fishery Co., Tokyo. Milk casein (Hammarsten type) and mineral or vitamin mixture (AIN-76 likeness) were products of Oriental Yeast Co., Tokyo. All other ingredients were commercially available.

of the HMF is considered nutritionally unavailable as a consequence of growth experiments (4). Sixty F-344 rats of ages of more than a half year were divided into 6 groups (n=10), among which 5 groups were provided with respective diets containing 0.2% sodium deoxycholate and one group was given a 10% milk casein diet exclusive of deoxycholate [casein (-DCA) group], housed individually in hanging cages in air-conditioned facilities for animal care, and allowed free access to the respective diets shown in Table 1. All of the rats received azoxymethane treatment once a week for the first 3 wk in the usual manner previously described (4, 5). During the feeding period, blood sampling and per-anal endoscopy were carried out on the rats under anesthetization at 6 wk intervals between the 10th and 34th wk. Incidentally, feces were collected for a few days in time to the interval of diet exchange just before anesthetization on the day of blood-gathering.

This experimental design was approved by the Animal Experiment Committee of Kyoto Prefectural University and the rats were cared for in accordance with the Guidelines Concerning the Care and Use of Laboratory Animals.

Endoscopic observation of the colon. Rats of each group were starved overnight after blood-gathering from the tail vein under etherization at stated times, followed by insertion of a bronchus fiberscope with a tip diameter of 3.0 φ mm (type BF-3C30, Olympus Co., Tokyo) over the range of 80% of the whole colonic length under anesthetization with pentobarbital. Endoscopic observation was repeated five times at intervals of 6 wk from wk 10 to wk 34.

Plasma bile acid, cholesterol and triacylglycerol determinations. The plasma that had been preserved in its frozen state after separation by centrifugation from blood samples was thawed and divided into small portions, which were used for the routine assay of plasma biochemical parameters. These plasma parameters were determined as previously described (4), according to the manufacturer's instructions using the respective assay kits (products of Wako Pure Chemical Co., Osaka), and the measured values were expressed in terms of μM or mM with deoxycholic acid, cholesterol and oleic acid as standards.

Quantification of fecal bile acid excretion. The feces of each rat were pulverized after lyophilization and weighed on a scale. A definite amount of feces powder (100 mg) was saponified at 70°C for 60 min with 10 volumes of 0.2 M NaOH in 90% ethanol and washed with petroleum ether to remove neutral steroids. Acidic steroids remaining in the aqueous layer were converted to free form by autoclaving at a strongly alkaline pH, and then separated by extraction with chloroform-methanol (2:1) at an acidic pH below 2. The amount of acidic steroids thus obtained was measured using the same assay kit for bile acid as mentioned above.

Statistical analysis. Data on plasma biochemical parameters and fecal bile acid excretion were expressed as means±SE. Multiple comparisons among groups were made according to the Student-Newman-Keuls test (12), the differences being judged significant at p<0.05. On the other hand, plasma bile acid concentrations were plotted against tumor incidence or fecal bile acid excretion at 28 and 34 wk, respectively, and regression equations and correlation coefficients between these two variables were analyzed on the basis of the least squares method.

RESULTS

Figure 1 shows changes in body weight during the feeding period with regard to the azoxymethane-treated rats of the 6 groups. The body weight gain through 34 wk was the highest in the codfish meat and casein (-DCA) groups, while no increase was observed for the milk casein and wheat gluten groups. The yolk protein and soybean HMF groups were ranked between the former and the latter. There were significant differences among these superior, intermediate and inferior ranks. The milk casein group was significantly inferior in food intake to the other groups, reflecting stagnant body weight gain in this group.

Figure 2 illustrates graphically the actual numbers of living and tumor-bearing rats in each group at 10, 16, 22, 28 and 34 wk. The development of tumors in the
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Colon was not observed at 10 or 16 wk, although a rat or two in groups other than the soybean HMF group died of either a mistake in insertion of the fiberscope or some unknown cause. On and after the 22nd wk, such operational mistakes were prevented, but the death of rats occurred unexpectedly in the ordinary duration of care. For such cases, the colon was immediately excised from the carcass and examined for tumor development. There were no cases, however, in which dead rats suffered from colonic tumor (data not shown). Tumor development was found at 22 wk only in the milk casein group, at 28 wk in groups other than the casein (−DCA) group and at 34 wk in all of the groups, even though the actual number of countable rats varied with the time and group. Tumor incidence (%) at 28 or 34 wk was estimated at 0.0 or 25.0, 11.1 or 33.3, 14.3 or 16.7, 22.2 or 44.4, 25.0 or 50.0 and 50.0 or 66.7 for the casein (−DCA), soybean HMF, yolk protein, wheat gluten, codfish meat and milk casein groups, respectively.

Table 2 summarizes the results of measuring plasma bile acid, cholesterol and triacylglycerol concentrations at 28 and 34 wk when tumor development was perceived by endoscopy in most or all of the groups. Plasma bile acid concentration was significantly low in the casein (−DCA), soybean HMF and yolk protein groups relative to the milk casein group at both 28 and 34 wk. A somewhat similar tendency to this was also observed for plasma cholesterol concentration with the exception of hypercholesterolemia in the casein (−DCA) group. Conversely, plasma triacylglycerol concentration was highest in the casein (−DCA) group but was exactly the same among the other groups. The hypocholesterolemic effect was a characteristic of the soybean HMF diet despite its supplementation with DCA, being in connection with bile acid uptake at a low level into the plasma.

Subsequently, the measured values in Table 2 were plotted against colonic tumor incidence at 28 or 34 wk. As shown in Fig. 3, two regression lines drawn between plasma bile acid concentration and tumor incidence provided the correlation coefficients of $r=0.994$ ($p<$...
Table 2. Plasma bile acid, cholesterol and triacylglycerol concentrations in rats fed the experimental diets at 28 and 34 wk.

| Dietary group        | Bile acid (μM)        | Cholesterol (mM)    | Triacylglycerol (mM) |
|----------------------|-----------------------|---------------------|----------------------|
|                      | 28 wk                 | 34 wk               | 28 wk                | 34 wk                | 28 wk                | 34 wk                |
| Milk casein          | 90±9                 | 96±19               | 3.2±0.1             | 3.5±0.2             | 1.1±0.1             | 1.2±0.3             |
| Soybean HMF          | 41±5<sup>bc</sup>    | 20±2<sup>c</sup>    | 1.8±0.2             | 1.8±0.1             | 1.0±0.2             | 0.8±0.1             |
| Yolk protein         | 44±4<sup>bc</sup>    | 28±5<sup>b</sup>    | 2.1±0.2             | 2.5±0.1             | 1.2±0.1             | 1.3±0.2             |
| Wheat gluten         | 51±5<sup>b</sup>     | 73±17<sup>ab</sup>  | 2.2±0.2             | 2.3±0.3             | 0.9±0.1             | 0.8±0.2             |
| Codfish meat         | 54±4<sup>b</sup>     | 52±13<sup>abc</sup>| 1.9±0.1             | 2.5±0.2             | 1.2±0.1             | 1.0±0.1             |
| Casein (-DCA)        | 29±3<sup>c</sup>     | 18±1<sup>c</sup>    | 2.5±0.2             | 2.4±0.1             | 2.5±0.2<sup>c</sup>| 3.0±0.3             |

Values are the means±SE; those without common superscripts in the same column are significantly different at p<0.05 by the Student-Newman-Keuls test.

DISCUSSION

There were no significant differences between two groups of 7-mo-old rats fed usual 10 and 20% casein diets for 8 wk, not only in nitrogen balance or body weight gain but also in plasma protein and lipid levels (13). In other words, the 10% casein diet was sufficient for the protein requirement of mature rats, unlike growing specimens. This is the main reason why the protein contents in the experimental diets were arranged to a rough 10% irrespective of their amino acid composition. For the duration of the tumorigenic experiments with mature F-344 rats, their body weights were really maintained without any considerable change despite the uptake of a 10% gluten diet insufficient in lysine. No variation in body weight gain was also observed for rats given the DCA-supplemented casein diet. Taking into account an approximate 1.3-fold increment in weight since 34 wk prior in the casein (-DCA) group, the above observation suggests that supplemental DCA may have caused a poor appetite. Alternatively, codfish meat was far more effective in raising body weight gain, prob-
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Fig. 4. Correlations between plasma bile acid concentration and fecal bile acid excretion at 28 and 34 wk. The individual amounts of bile acid excreted into the feces were obtained daily, for an average of 3 d, before blood-collecting, and were plotted against their corresponding bile acid concentrations in the plasma. Two regression lines with high correlation coefficients were drawn among 5 groups except the casein (−DCA) group. White square, groups at wk 28; black circle, groups at wk 34.

ably body fat accumulation, relative to milk casein under DCA-supplemented conditions. It is highly possible that the taste and/or flavor of fish meat caused a good appetite. Yolk protein and soybean HMF also brought about some increment in weight, although being inferior to fish meat. The increment may possibly be explained by DCA masking rather than in nutritional aspects, as will be described later in more detail.

Although we could get along without serious attention to the differences in weight among the dietary groups, one or a few rats in each group died at the age of one year or more, as we apprehended. Some deaths at early stages were caused by inexperience in performing endoscopy, but not in the latter half of the feeding period. We can at least safely say that death was not due to tumorigenesis because of the absence of tumors in the colon of those rats which died accidentally. Unfortunately, air-conditioning trouble took place on a hot August day just before the 40th wk and many rats died at once due to the sweltering heat. Continuance of the feeding was brought to a close at this point in time.

Animals, from which the colons were excised to inspect for tumor incidence, admit of no survival. In this connection, we had much interest in a recent report (14) that endoscopy was available for evaluating the preventive effect of wheat bran against experimentally induced colonic tumorigenesis. Accordingly, a fiberscope with a 3 mm Ø tip specific for human bronchi was introduced into our tumorigenic experiment. We are convinced that we have obtained a useful piece of information on tumor incidence from endoscopic observations at 6 wk intervals until the 34th wk, though we were obliged to give up our objective experiment at the halfway point. Colonic tumor was first found in 2 rats of the milk casein group at 22 wk, and occurred frequently in the same group later on. The number of living rats at 34 wk fell to six, of which two-thirds were tumor-bearing. The other groups also ranged in number from 6 to 9 at 34 wk. As for the tumor-bearing ratios for living rats in each group, they tended to be low in the soybean HMF and yolk protein groups as compared with the wheat gluten and codfish meat groups, let alone with the milk casein group. Soybean HMF is famous for its high bile acid-binding and -excreting capacities (1-3). Egg yolk protein deprived of lipids under mild conditions (15) is naturally well-mixable with lipids, or rather “it adsorbs steroids sufficiently.” Both soybean HMF and yolk protein have something in common with each other in this respect. An attempt to correlate plasma bile acid concentration with colonic tumor incidence is based on this speculation. Two regression lines with high correlation coefficients at 28 and 34 wk (Fig. 3) suggest the possibility that tumorigenesis undergoes promotion by hemal bile acids from the basolateral side rather than by luminal bile acids from the apical side.

With the exception of the casein (−DCA) group, there was a reverse relationship between fecal bile acid excretion and plasma bile acid concentration (Fig. 4). That is, augmentation in the bile acid excretion into the feces is reflected in the plasma concentration of bile acids. Above all, soybean HMF and yolk protein were effective in decreasing the plasma bile acid concentration (Table 2) though the amount of daily DCA intake was
much more in the soybean HMF or the yolk protein group than in the milk casein group (Fig. 1). As a matter of course, both fecal and plasma bile acids were at low levels in the casein (-DCA) group. Data on this group, nevertheless, strays from the relationship between fecal and plasma bile acids. It thus seems reasonable to consider that plasma bile acid concentrations are more intimately involved in tumor incidence. Needless to say, attention must be paid to changes in microflora under dietary conditions used in this experiment, but the problem was left untouched. In any event, further investigation on the cancer-promotive action of secondary bile acids is required in order to interpret the present findings correctly.

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