High Protein Intake Promotes the Growth of Hepatic Preneoplastic Foci in Fischer #344 Rats: Evidence that Early Remodeled Foci Retain the Potential for Future Growth

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ABSTRACT The effects of successive administration, withdrawal and readministration of high protein diets (20% casein) on the promotional growth, remodeling and regrowth of aflatoxin B1-induced preneoplastic liver lesions (foci) were examined. Weanling male Fischer 344 rats were given 10 intragastric doses of aflatoxin B1 at a level of 250 μg/kg body weight over a 2-wk dosing period (initiation). The subsequent 12-wk period was subdivided into four feeding periods, each lasting 3 wk (promotion). Two groups of rats were fed either a 20 or 5% casein diet during all four periods; additional groups were alternately fed these diets in different sequences. Switching from the high protein diet to a low protein diet (5% casein) resulted in marked remodeling (regression) of the growing lesions to a response level similar to that in animals that did not receive the initial promotional stimulus of high protein feeding. However, refeeding the high protein diet caused significant reappearance of these lesions. The restimulated development of these remodeled lesions far exceeded lesion growth in animals receiving only the late promotional stimulus of high dietary protein. Thus, these data suggest that a second occurrence of high protein feeding promotes the growth of remodeled foci, thus demonstrating their potential for future promotional growth. J. Nutr. 121: 1454-1461, 1991.

INDEXING KEY WORDS:
- rats
- carcinogenesis
- protein
- γ-glutamyltranspeptidase foci

Current carcinogenesis theory states that carcinogen-altered cells evolve through three stages of alteration—initiation, promotion and progression—before becoming tumors (1). Initiation involves damage to the DNA either directly by carcinogen binding, or indirectly through diverse biological reactions such as oxidative DNA damage. Initiation is considered irreversible. Alternatively, promotion is considered readily reversible and is characterized by sustained and unrepaired growth of cells containing damaged DNA to form foci or clusters of cells presumably arising by clonal expansion of single initiated cells. Progression includes the final stage of tumor development, followed by host death. Unlike promotion, very little is known about the reversibility of progression.

Epidemiological studies suggest that high protein diets (particularly those high in animal proteins) are a risk factor for selected cancers, including colorectal and breast cancer (2). In animal studies, low protein diets have been shown to inhibit carcinogenesis induced by various carcinogens in diverse tissues of several species (3-5). Although dietary protein is known to modify the formation of DNA adducts (6) and influence aflatoxin B1(AFB1)-induced carcinogenicity (5), this nutrient is believed to have greater influence over post-initiation lesion development (7).

Our group has undertaken numerous studies to explore the effect of dietary protein on the growth of AFB1-induced γ-glutamyltranspeptidase positive (GGT+) foci in the liver of Fischer 344 rats. GGT+ foci have been regarded as indicators of preneoplastic growth. In this model, our studies have indicated the following: 1) the effect of dietary protein on carcinogenesis is more pronounced during promotion than during initiation (7); 2) GGT+ foci development increases sharply at protein intake levels just above dietary requirement (8); 3) protein intake during promotion is more rate-limiting for preneoplastic lesion development than is the total dose of carcinogen initially administered (9); 4) sustained development of GGT+ foci is dependent on a high level of protein intake.
intake (10), 5) GGT+ foci and liver tumors develop in dose dependence to the level of protein intake, and inhibition by consumption of low protein diets occurs in spite of increased energy intake (11), and 6) GGT+ foci are reliable markers of neoplastic potential in this model of carcinogenesis (11). Thus, on the basis of this evidence, we contend that these foci are preneoplastic, not only presumptively preneoplastic.

A majority of the GGT+ foci (approximately 85–95%) in other experimental models will remodel or regress to become phenotypically normal in appearance following the initiation period (12). Some studies (13) suggest that preneoplastic foci remodel upon withdrawal of carcinogen exposure, whereas other studies (14) suggest that foci remodel upon withdrawal of a promotional stimulus. The latter study (conceptually similar to our own) showed that the number of enzyme-altered foci in rat liver decreased sharply after the withdrawal of the promoter phenobarbital, whereas the volume of tissue occupied remained unchanged or increased slightly (14). However, upon readministration of phenobarbital, foci reappeared either at the same or at an increased level when compared with the level observed before withdrawal of the promoter. These data suggest that, despite a phenotypically normal appearance upon withdrawal of the promoter, remodeled foci retain the potential for future growth.

Unlike phenobarbital (14), phorbol esters (15) or partial hepatectomy (16), high protein intake has not been typically considered a promoter of preneoplastic or tumor growth. Nonetheless, increased protein intake induces the following responses typical of promoters: 1) enhancement of cell proliferation as indicated by mRNA levels of plasma proteins (17) and RNA/DNA synthesis and metabolism (18); 2) increased ornithine decarboxylase (EC 4.1.1.17) activity (19); 3) increased focal GGT levels (7–11); and 4) increased tumorigenesis (3–5, 11, 19). These responses are typical of the more traditional promoters (15, 20, 21). Furthermore, Williams (20) classifies as a tumor promoter an agent that permits tumor formation by altered cells that would otherwise remain dormant; thus, high protein intake has been shown to enhance tumorigenesis (11, 19), whereas low protein intake permits foci and tumors to remain dormant (10, 11). These observations suggest that high protein intake can be regarded as a strong promoter of carcinogenesis at typical levels of intake.

As a promoter, high protein intake could stimulate the development either of the so-called persistent foci, some of which are presumed to progress to the formation of hyperplastic nodules and tumors (22, 23) and which are often generously and uniformly stained to indicate the presence of GGT, or of the remodeled foci, which are sometimes irregularly stained and can revert to tissue morphologically more normal in appearance (22). There is some indication that the remodeling process may include apoptotic removal (or death) of the GGT+ cells (24), thus remodeled foci would be eliminated that could have been recruited for further development by high protein feeding. Data from Kitagawa (25) suggest that remodeling could occur by replacing abnormal cells in each focus with encroaching normal cells. Alternatively, Williams and Watanabe (22) suggested, using a system employing foci resistance to iron accumulation, that the phenotypic remodeling loss of this property involves a much more gradual process of cell repair.

The present study was designed to evaluate the potential for remodeled preneoplastic foci to renew growth in the presence of the promotional stimulus of high dietary protein. This was undertaken by feeding a high protein diet to two groups of rats with similar lesion yields, including one group with no previous stimulation of foci growth and a second group with previously stimulated foci growth that was later remodeled by low protein feeding.

### MATERIALS AND METHODS

**Animals and diets.** Weanling male Fischer 344 rats (Charles River, Kingston, ME) with average body weights of 40–60 g were randomized into treatment groups. Animal care was in accordance with Cornell University institutional guidelines. Rats were fed an AIN-76A diet (26) providing 20% casein during the 2-wk acclimation predosing period, 2-wk dosing period (initiation), and the 1-wk postdosing AFB clearance period. During the 12-wk promotion period of dietary treatment, animals were fed an AIN-76A diet providing either 20 or 5% casein (which is 17.4 or 4.35% protein because casein is 87% protein) with sucrose and corn starch being substituted proportion-
FIGURE 1 Experimental protocol showing aflatoxin B₁ (AFB₁) dosing period (initiation) and dietary casein treatments for groups killed at 3, 6, 9 and 12 wk (promotion). A group designated 20:5, for example, was given 20% casein during wk 1–3, 5% casein during wk 3–6, then killed at the end of wk 6. In total there were 16 groups (14 AFB₁ groups, two non-AFB₁ groups), n = 8–10 rats/group.

FIGURE 2 Body weights [mean ± SEM] for rats fed 20:20:20:20 and 5:5:5:5 dietary casein. Body weights for rats fed 5 or 20% casein were significantly different at P < 0.01 as determined by separate Student’s t test comparisons at 3, 6, 9 and 12 wk post-initiation; n = 8–10 rats/group at each time point.

Histology and preneoplastic lesion quantification. Rats were anesthetized and decapitated. Multiple slices of the median liver lobe were then immediately frozen on dry ice and subsequently stored at −85°C. Liver slices were embedded in O.C.T. compound (American Scientific Products, Rochester, NY) and multiple adjacent frozen (−17°C) cryostat sections were taken with a Cryocut II cryostat (American Optical, Buffalo, NY). Adjacent sections (10 μm) were then fixed and stained with hematoxylin and eosin, for morphology/pathology determination and also for elevated levels of γ-glutamyltranspeptidase enzyme (GGT) (EC 2.3.2.2) by the method of Rutenberg et al. (27).

GGT+ foci were examined under a Nikon (Nikon, Tokyo, Japan) phase contrast light microscope. GGT+ foci in each liver section were counted and measured in two directions with an eyepiece micrometer. Foci diameters were determined directly from micrometer measurements. Liver section areas were quantified by projecting images of the liver sections with a photographic enlarger at constant magnification. Images were traced on paper, cut out, weighed, and the weight was divided by that of a standard 1-cm² image projected at the same magnification. Foci data were calculated by stereological formulae as described by Campbell et al. (28).

Statistical analysis. Foci responses among the treatment groups were compared by the nonparametric rank sum (one-tailed) of Wilcoxon and Wilcox (29). Body weights and food consumption data for the 5:5:5:5 and 20:20:20:20 groups for each kill time (i.e., 3, 6, 9 and 12 wk) were compared by the Student’s t test (two-group comparison of the 5% casein-fed rats with the 20% casein-fed rats) using Minitab (30).
TABLE 2

Food consumption by rats fed 5 or 20% casein diets throughout the 12-wk study period

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Weeks 1-3</th>
<th>Weeks 3-6</th>
<th>Weeks 6-9</th>
<th>Weeks 9-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:5:5</td>
<td>17.5 ± 0.4</td>
<td>15.7 ± 0.5</td>
<td>17.9 ± 0.8</td>
<td>17.7 ± 0.8</td>
</tr>
<tr>
<td>20:20:20:20</td>
<td>15.3 ± 0.5</td>
<td>13.9 ± 0.5</td>
<td>15.4 ± 0.6</td>
<td>15.8 ± 0.7</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for 8-10 rats per group. Food consumption between 5 and 20% casein-fed groups at 3, 6, 9 and 12 wk as determined by the Student's t test at each time point was significantly different at P < 0.05. Although 5% casein-fed rats were smaller than 20% casein-fed rats [see Fig. 2], they consumed more food. However, protein intakes (when corrected for body weights) were significantly greater (P < 0.01) by the Student's t test for the 20% casein-fed groups.

RESULTS

Body weights are presented in Figure 2. Animals fed the 5% casein diet were significantly smaller than animals fed the 20% casein diet throughout the dietary treatment periods (P < 0.01). Aflatoxin B1 treatment had no effect on body weight [data not shown]. When animals were switched from the 20% casein diet to the 5% casein diet they grew more slowly; switching from the 5% casein diet to the 20% casein diet caused more rapid growth [data not shown].

Food consumption data are shown in Table 2. The 5% casein-fed rats consumed more food than the 20% casein-fed rats throughout the study period despite the facts that the 5% casein-fed rats were smaller and the two diets were isoenergetic. When animals were switched from the 20% casein diet to the 5% casein diet, they began to consume more food; the opposite was also true [data not shown].

Foci development at 3, 6, 9 and 12 wk for animals fed either the 5% casein diet or the 20% casein diet throughout the promotion period are shown in Figure 3. As in our previous studies (7-11), rats fed lower protein diets developed significantly fewer foci than did rats fed higher protein diets. Foci development was assessed by calculation of the number of GGT+ foci/cm² liver tissue [data not shown], number of GGT+ foci/cm³ liver tissue, average diameter of GGT+ foci [data not shown] and the percentage of liver volume occupied by foci [a calculated parameter]. Each of these measurements supports the conclusion that the lower casein diets inhibited foci development despite greater energy intake.

Figure 4 indicates that foci development in rats receiving an early promotional stimulus [high dietary protein for 3 wk] followed by an inhibitory intervention [low dietary protein for the subsequent 3 wk] was reduced to a level similar to that in animals receiving no early promotional stimulus [comparison of 20:5 with 5:5 group]. In other words, hepatic foci in the 20:5 group underwent significant remodeling during the low protein intervention, lesion development, at least phenotypically, was equivalent in the 5:5 and 20:5 groups. However, re-introduction of the high dietary casein promotional stimulus indicated that these remodeled foci retained the potential for greater promotional growth [comparison of 20:5:20 with 5:5:20 group]. That is, at 9 wk post-initiation, foci development was significantly greater (P < 0.05) in animals receiving the early promotional stimulus and then remodeled [20:5:20] than it was for animals not receiving this early stimulus [5:5:20].

The data presented in Figure 5 further support this observation. First, despite significant foci growth at 6 wk in the high protein group as compared with the low protein group [comparison of 20:20 with 5:5 group], subsequent intervention with low protein diets markedly reduced foci response to a level phenotypically near [and statistically equivalent to] the foci response in animals receiving no early promotional stimulus [comparison of 20:20:5 with 5:5:5 group]. Then, re-introduction of the promotional stimulus of high protein feeding stimulated greater foci de-
development among animals that had experienced early lesion growth (comparison of 20:20:5:20 with 5:5:5:20 group). Thus, high dietary protein stimulation of GGT+ foci development was significantly greater in animals with growing lesions that were remodeled than in animals whose lesions remained dormant. These results suggest that remodeled preneoplastic foci retain an enhanced potential for future growth.

**DISCUSSION**

Various lines of evidence support the conclusion that GGT+ foci growth reliably identifies the presence of preneoplastic and/or abnormal tissue. The evidence includes the following: 1) the suggestion by Hanigan and Pitot (31) that cells possessing higher levels of GGT enzyme have a selective advantage over normal hepatocytes for continued growth (thereby inferring that reductions in GGT enzyme activity would reduce growth advantage); 2) the finding of Sinha et al. (32) that ras-induced transformed clones stained positive for GGT whereas non-transformed clones stained negative for GGT; 3) the observations of Benedetti et al. (33) from serial liver sections showing that foci that had lost susceptibility to lipid peroxidation (a feature common to tumor cell membranes) closely overlapped areas staining positive for GGT; 4) the findings of Williams et al. (34) that cells from GGT+ adenomas were enlarged and the nuclei were irregular whereas cells from adenomas that were GGT- were pathologically more normal, and 5) the observations that the majority of human hepatocellular carcinoma are GGT+ (35). However, the best evidence that GGT+ foci are reliable indicators of preneoplasia (with the potential to become neoplastic) in this carcinogenesis model comes from a recently completed tumor study in our laboratory, in which the correlation between GGT+ foci development and ultimate tumor yield was very high ($r = 0.98$) [11]. In addition, we repeatedly found that serial liver sections stained with hematoxylin and eosin indicated areas of altered morphology that are congruent with focal GGT+ areas; therefore, we do not believe that these observed changes in foci development merely represent temporary alteration in cellular levels of GGT enzyme. Rather, we have evidence of substantive changes in preneoplastic development.

Along with the results of our earlier studies [7–11], these data indicate that hepatic preneoplastic foci development can be markedly inhibited by diets con-
taining lower dietary protein. In addition, even though these foci have remodeled or have lost those properties that make them phenotypically identifiable, they retain a memory of their altered state and can be recalled to grow in earnest with appropriate stimulation, i.e., they retain the potential for future promotional growth.

These data may call into question the assumption that it is predominantly the persistent foci (those that do not remodel back to normal-appearing tissue) rather than the remodeled foci that are capable of progressive growth eventually leading to hepatocellular carcinomas (1). Although it is thought that appropriate stimuli can induce even remodeling foci to grow (16, 36), some data suggest that remodeling foci are less likely than persistent foci to grow and become tumors (1). However, data from the present study suggest that, during exposure to an appropriate nutritional stimulus, foci that have been stimulated, then remodeled, then re-stimulated, can be induced to renew their growth to a level nearly equivalent to foci that have been continuously stimulated. For example, foci development in the 20:20:5:20 and 20:20:20:20 groups was 0.083 ± 0.017 and 0.088 ± 0.024 percent liver volume, respectively.

There are three possible outcomes for preneoplastic clonal growths: continued growth, dormancy or death. Key determinants of the extent to which un-repaired focal growth may be sustained are the promotive environment and/or nutritional milieu in which the tissue exists and the length of exposure to that environment. In this study, intervention with low protein feeding may not have continued long enough for complete repair of the lesion and a permanent shift in foci development.

It has been suggested that during hepatocarcinogenesis, preneoplastic lesions become increasingly distinguishable from normal tissue (16, 36-39) and that remodeling of these abnormal lesions can occur either by replacement, preferential death or repair. Kitagawa (25) suggested that abnormal cells in remodeling foci are replaced by normal cells, whereas Bursch et al. (24) suggested that remodeling may occur by greater cell death within foci than in normal tissue. In contrast, Williams and Watanabe (22) found that, in the remodeling process, cells in foci that are resistant to iron accumulation only slowly reverted back to iron storing cells with abnormal morphology and then to cells with more normal morphology. Data from this study support the latter view of remodeling, in which cells within the focus gradually are repaired rather than by being replaced by other cells or by preferential death. The view that remodeling most likely occurred by cell repair is supported by the fact that renewed consumption of the high protein diet enhanced greater foci growth in animals with remodeled foci than in animals with previously unstimulated foci. Because no additional DNA damage was incurred to create new foci, this enhanced focal growth most likely evolved from re-stimulated growth of repaired or remodeled cells within each focus.

Although quantification of several marker enzymes for preneoplasia may have added confidence to these data, we believe that the basic finding (i.e., high protein intake promotes the growth of enzyme-altered preneoplastic foci) would have been the same, based on the lines of evidence cited in the first paragraph of this discussion.

Sidransky et al. (40) also quantified the growth of GGT+ enzyme-altered foci and found that supplementation of a choline-sufficient diet with the single amino acid tryptophan increased the development of GGT+ hyperplastic foci in diethyl-nitrosamine-induced Sprague-Dawley rats. They concluded that increased dietary tryptophan has a promoting effect on liver carcinogenesis. However, we do not believe that the promotion effect of high protein diets can be traced to any particular amino acid, even though the 5% casein diet included only one-quarter of the tryptophan content contained in the 20% casein diet. In support of this view, we recently found that GGT+ foci development was markedly reduced by feeding a 20% soy protein diet (11), which has a higher tryptophan content than does the 20% casein diet.

Nevertheless, the levels of the constituent amino acids composing proteins (or their amino acid balance) are determinants of tumorigenesis potential. A review of the early work on the tumorigenic effect of varied dietary proteins in animal carcinogenesis models revealed that the inhibition of tumor development was restored by supplementing these diets with whichever amino acids were most deficient (41). Also, in a recent study (42) by our group, GGT+ foci development, when inhibited by the feeding of a 20% gluten diet, was restored by the addition of the deficient amino acid lysine. Collectively, these findings suggest that both the type of dietary protein (or, alternatively, the amino acid balance) and the level of protein intake play critical roles in the promotional growth of enzyme-altered foci. In support of this viewpoint, other investigators using various carcinogens as inducers, examining diverse tissues and using different animal strains have also reported increased preneoplastic and neoplastic development in animals fed high dietary protein (3-5, 19).

It is not likely that the compensatory decrease in carbohydrate intake that accompanies the increased protein intake was responsible for the enhanced development of GGT+ foci. First, protein intake was increased by 300%, whereas carbohydrate intake was decreased only by 17%. Thus, the relative change in protein intake was far more dramatic than the relative change in carbohydrate intake. Second, we have observed that an increase in sucrose intake, when compared with mixed sugars, increased GGT+ foci.
development (43), an observation in accord with the report of Hei and Sudilovsky (44). Thus, any effect of sucrose would have diminished the inhibitory effect of a low protein diet, an observation strengthening our suggestion that low protein intakes inhibit GGT+ foci development.

Although a 5% casein diet is not generally considered nutritionally adequate [i.e., it does not support maximal growth], for every health index we have thus far measured, the 5% casein diet supports better health in rats than does the 20% casein diet.

These data, along with our previous studies (7–11), demonstrate that a high intake of dietary protein is a critical determinant of preneoplastic foci development and growth. The mechanism by which dietary protein promotes this growth is not understood, but it is probable that a large number of mechanisms are operating simultaneously. One of the more important determinants may be that elevated intakes of dietary protein stimulate cell proliferation, which in turn enhances carcinogenesis (45, 46). Ames (47) recently hypothesized that because "normal" DNA damage occurs at rates far higher than what would be required for tumor development, alterations in cell proliferation rates may be more predictive of tumorigenicity. In addition to its effect on cell proliferation, dietary protein also may affect lesion development indirectly by its effect on energy utilization and expenditure (as illustrated in these data by the higher energy intakes in the animals fed low protein diets), immune surveillance, cholesterol metabolism, hormonal status, etc. Further work is required to elucidate these mechanisms.

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LITERATURE CITED


