Demonstration of the Need for End Point Validation of Putative Biomarkers: Failure of Aberrant Crypt Foci to Predict Colon Cancer Incidence

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Abstract

Seven-week-old Sprague-Dawley rats were fed a semipurified AIN76 diet and were given a weekly injection of the colon carcinogen 1,2-dimethylhydrazine for 8 weeks (initiation stage of carcinogenesis). The rats were divided into seven groups and each group of rats was placed on one of seven different modifications of the AIN76 diet for the next 24 weeks (promotion stage of carcinogenesis). The mean numbers of aberrant crypt foci/rat and the incidence of adenocarcinomas from some of the seven dietary groups were found to be significantly different. However, all attempts to show a significant correlation between the mean number of aberrant crypt foci/rat and the incidence of adenocarcinomas failed. Therefore, the number of aberrant crypt foci/rat cannot by itself be used as a reliable quantitative predictor (biomarker) of the efficacy of dietary intervention or of chemopreventive procedures on modulating the risk of developing colon cancer. This conclusion emphasizes the need for end point validation of potential cancer biomarkers before the biomarkers can be considered predictive of modulation of the risk for colon cancer.

Introduction

Enlarged, hyperplastic (aberrant) colonic crypts can be easily identified in methylene blue, Giemsa or toluidine blue stained whole mount preparations of colon from humans with colon cancer (1) and from rats which have been treated with a chemical carcinogen (2-4). Single aberrant crypts or ACF (2 or more aberrant crypts) were not found in the colons of rats which had not had treatment with a colon carcinogen (5) and ACF have been shown to increase in a dose dependent manner in response to chemical carcinogens (1, 2). ACF are found mainly in the descending colons of carcinogen treated rats (3, 5, 6) and are much more frequent in the left colon than in the right colon of humans (4). Histological and cytochemical studies in rats show disturbances in the differentiation patterns and enzyme activities of the cells in ACF and these disturbances are similar to those found in neoplastic tissue (3, 7, 8). These findings have led investigators to the conclusion but have not proven that ACF represent preneoplastic stages in the development of adenocarcinomas in the descending colon of humans (1) and in carcinogen treated rats (2-10). It can be hypothesized from these observations that procedures which increase or decrease the numbers of ACF/rat will increase or decrease the risk for colon cancer. This report contains findings on the relationship between the number of ACF and the incidence of adenocarcinomas in the descending colon in groups of rats that were placed on one of seven different dietary interventions during the promotional stage of colon carcinogenesis. Briefly stated, specific dietary interventions were shown to alter both the average number of ACF/rat and the incidence of adenocarcinoma; however, the number of ACF/rat was not predictive of the incidence of adenocarcinoma. Based on these findings, it must be concluded that the number of ACF present in the ascending colon is not by itself a reliable predictor of the efficacy of dietary interventions at reducing the incidence of colon cancer.

Materials and Methods

Animals. Four- to six-week-old male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley. Cages had solid plastic sides with high, wire mesh false bottoms to minimize access to the bedding or feces. The animal housing room was well ventilated (20 air changes/h) with a temperature of 25°C and automatically controlled light/dark of 14/10 h. All animals had ad libitum access to food and deionized water during the entire experiment.

Diets. Compositions of the diets (Table 1) were based on the standard AIN-76 formula with the substitution of guar gum or pectin for the cellulose and alteration in the corn oil content of the pectin diets. The diets were formulated so that each kcal of food consumed would provide an equal quantity of protein, complex carbohydrates, vitamins, and minerals.

Experimental Design. Upon receipt, the animals were randomly paired, assigned cages, and ear marked for identification. Ten days were allowed for adjustment to their new environment.

Pairs of rats were then randomly divided into two groups. One group received DMH and the other group received only the saline vehicle. DMH solutions (26.6 mg 1,2-DMH dihydrochloride/ml of solution made up in 0.9% saline and 0.18% EDTA and then pH adjusted to 6.5 with NaOH) were freshly prepared each week. Rats received s.c. injections of 12 mg DMH base/kg body weight (0.1 ml of solution/100 g body weight) once each week for 8 weeks (defined as the initiation stage of the experiment). Control animals received an equivalent volume of the vehicle (0.9% saline and 0.18% EDTA). All animals were housed in the same controlled access room during the entire experiment. During the adjustment and initiation periods, all rats received standard AIN-76 formula food [with 5% cellulose (Alphacel) and 5% corn oil] and deionized water.

At the beginning of week 9, the pairs of rats (with or without DMH treatment) were randomly subdivided into groups to be placed on the different diets (see Table 1). Weeks 9-32 of the experiment were defined as the promotional stage of the experiment.

Tissue Preparation and Analyses. The rats were ether anesthetized and then killed by decapitation, and a gross pathological examination was performed. Ears, heart, lungs, liver, spleen, kidneys, and small and large intestines were checked for tumors. The colon was removed, opened longitudinally, and carefully examined for tumors and tumor-like lesions. Descending colons were pinned flat, serosal side down, onto corkboard and then fixed in 10% buffered formalin. The tumor or lesion size (area) and distance from the cecum (if tumor was present in the large intestine) or stomach (if tumor was present in the small intestine) were measured and recorded. The tumor was removed, fixed in formalin, and then processed for histology. Four-μm-thick serial sections of the entire tumor were cut, mounted on glass slides and stained with hematoxylin and eosin for histological analysis.

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3 The abbreviations used are: ACF, aberrant crypt foci; DMH, dimethylhydrazine; SNK, Student-Newman-Keuls.
stained with hematoxylin and eosin. Microscopic examination was used to classify each tumor as an adenoma or an adenocarcinoma.

Staining and Counting of Aberrant Crypt Foci. Giemsa stain (3 ml of concentrate in 50 ml of phosphate buffered saline, pH 7.1) was used to aid visualization of the ACF. Descending colons were removed from the cork and placed luminal side up onto a numbered Petri dish. For ease in counting the ACF in 2 cm of colon length, each dish had a 2-cm section marked on the bottom with permanent ink. The colon was flooded with stain. After 30 min, excess stain was rinsed off with phosphate buffered saline. The Petri dish with the colon was then placed under a stereomicroscope (×30) for counting of ACF. The ACF were classified as small (1–3), medium (4–6), or large (>6) by number of crypts per foci. The number of ACF in each category in a section of the descending colon from 2.5 to 4.5 cm above the level of the pelvic rim and the full width of the opened colon was recorded. The width of the colon section was measured and recorded for calculation of the area in which ACF were counted.

Statistical Analyses. Differences in tumor incidence (i.e., the number of rats with a tumor/number of rats in the group) were evaluated by a binomial test. This nonparametric test compares the observed frequency in each category of a dichotomous variable with the expected frequency under a binomial distribution. The variable is tabulated into two categories based on where the dividing value between the categories is specified. The observed proportion is then compared to the test proportion (in this case the percentage of rats without tumors on a different diet) and a significance test is performed (12). If $P$ (probability that the observed and test proportion differ from the same distribution) for a comparison was $\leq 0.05$, the tumor incidence of the two groups was judged to be significantly different.

The total number of ACF/rat was calculated as the sum of the small, medium, and large ACF. The relationship between colon cancer incidence and ACF was examined in three different ways: (a) least squares linear regression analysis was used to test the correlation between the incidences of cancer in the descending colon and either the mean total number of ACF or the mean number of small, medium, or large ACF in each of the seven different feeding groups; (b) data from each feeding group were divided into rats which did or rats which did not develop adenocarcinomas. Two-way analysis of variance was used to determine statistical differences between the mean number of ACF/rat in rats with and in rats without adenocarcinomas in the seven different feeding groups; (c) data from DMH treated rats of all seven feeding groups were pooled. Probit analysis was used to determine whether the number of ACF could be used to predict the incidence of colon cancer. Probit analysis is appropriate for analysis of dosage effect on a quantal (all or nothing) response model (13). Thus, this analysis would answer the question: Can the number of ACF in a rat predict the occurrence of adenocarcinoma in the rat? The probit analysis produces a Pearson goodness of fit $x^2$; if the $x^2$ is $\leq 1.96$, the correlation is significant.

**Results**

The descending colons of rats which did not receive DMH (referred to as control rats) were examined. No ACF were found in the colons of control rats and none of these rats developed an adenocarcinoma. Therefore, no data from control rats are shown in this report. The width and length of all descending colons was nearly the same (range, 1.2–1.3 cm wide and 9.8–10.0 cm long) in all rats; thus aberrant crypts were counted in equal size areas and from an equal proportion of the colon. ACF were counted in the same location (2.5 to 4.5 cm above the level of the pelvic rim) in the descending colon in all rats.

The incidence of adenocarcinoma in the descending colon (Table 2) of the group of rats which consumed a diet formula containing 10% pectin/5% corn oil was significantly lower than the descending colon cancer incidence in the group of rats which consumed a diet formula containing 0% fiber ($P < 0.02$, binomial test). The adenocarcinoma incidence in the descending colon of the group of rats with 10% pectin/20% corn oil in their diet was significantly lower than the incidence of cancer in the descending colon of all other dietary groups of rats ($P < 0.0007$, binomial test).

One-way analysis of variance (Table 2) was used to compare the number of ACF/rat by dietary feeding groups. The groups of rats consuming 10% pectin with 10 or 20% corn oil had significantly fewer total ACF than all other groups of rats (SNK range test, $P \leq 0.05$). There was no significant difference in the number of large size (>6 crypts/foci) ACF/rat between the dietary groups of rats. The groups of rats consuming 10% pectin with either 10 or 20% corn oil had significantly fewer medium size (4–6 crypts/foci) ACF/rat than the groups consuming either 0% fiber, 5% guar gum, or 5% guar gum/5%...
Consuming 0% fiber was found to have significantly more small rats on all other diets, \( P < 0.04 \). Binomial test.

Which did not develop adenocarcinoma (Table 3, column means) showed that there was no difference in the total number of small ACF/rat present in rats which did or in rats which did not develop an adenocarcinoma. However, there was a significant decrease in the total mean number of ACF/rat in the descending colon in the groups of rats which consumed either 10% pectin/10% corn oil or 10% pectin/20% corn oil compared to the group which consumed the 0% fiber diet (Table 3, row means, one-way analysis of variance, SNK, \( P < 0.05 \)).

Fig. 1 shows the ACF and colon cancer incidence for the total population of rats. The rats were divided into quintiles by number of ACF and then the colon cancer incidence of each quintile was calculated. Probit analysis of the total population of DMH treated rats (using the raw data so that all the data points could be included) showed that there was not a significant correlation between the number of ACF present in a rat and the incidence of colon cancer; i.e., the number of ACF could not be used to predict the incidence of colon cancer. The Pearson goodness of fit \( \chi^2 \) was 176.04, df = 174, fit to the model \( P = 0.443 \). The \( \chi^2 \) is >1.96; therefore there is not a significant correlation between the number of ACF and colon cancer incidence. \( P = 0.443 \) shows that there was not a significant deviation of the data set from the model; therefore probit analysis was appropriate for this data set.

Discussion

Investigators (1-10, 14) have counted the number of ACF in rats 2 to 6 weeks after treatment with a carcinogen known to induce colon cancer. These past studies were designed to dem-

Table 2 Incidence of adenocarcinoma and aberrant crypt foci/rat in the descending colon of DMH treated rats maintained on one of seven different modifications of a semipurified AIN-76 diet * fed during the promotional stage of colon carcinogenesis

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Adenocarcinoma incidence</th>
<th>ACF/rat (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Large</td>
</tr>
<tr>
<td>0% fiber</td>
<td>0.47</td>
<td>50.4 ± 5.3</td>
</tr>
<tr>
<td>5% GG</td>
<td>0.33</td>
<td>42.4 ± 5.6</td>
</tr>
<tr>
<td>10% GG</td>
<td>0.33</td>
<td>36.4 ± 5.0</td>
</tr>
<tr>
<td>5% GG/5% P</td>
<td>0.37</td>
<td>39.2 ± 5.4</td>
</tr>
<tr>
<td>10% P/5% CO</td>
<td>0.23*</td>
<td>33.9 ± 3.9</td>
</tr>
<tr>
<td>10% P/10% CO</td>
<td>0.37</td>
<td>29.1 ± 4.4*</td>
</tr>
<tr>
<td>10% P/20% CO</td>
<td>0.06*</td>
<td>30.4 ± 2.7*</td>
</tr>
</tbody>
</table>

* Significantly greater than the mean ACF/rat of all other groups, \( P < 0.05 \), analysis of variance, SNK.

* Significantly less than the incidence of adenocarcinomas in rats on the 0% fiber diet, \( P < 0.0007 \), and significantly less than the incidence of adenocarcinomas in rats on all other diets, \( P < 0.04 \), binomial test.

* Significantly greater than the mean number of small ACF/rat of all other groups, \( P < 0.05 \), analysis of variance, SNK.

* Significantly less than the mean number of ACF/rat of rats on the 0% fiber diet, \( P = 0.04 \), analysis of variance, SNK range test.

* Significant greater than the mean number of ACF/rat of all other groups, \( P < 0.05 \), analysis of variance, SNK.

° Aberrant crypt foci were counted in a 2-cm-long section of descending colon from 2.5 to 4.5 cm above the level of the pelvic rim and the full width of the opened descending colon (1.2-1.3 cm). Small ACF had 1-3 crypts/foci, medium ACF had 4-6 crypts/foci, and large ACF had >6 crypts/foci.

° See Table 1 for composition. GG, guar gum; P, pectin; CO, corn oil; \( n = 26-28 \) rats/group.

° Number of rats with adenocarcinoma in the descending colon/total number of rats in each dietary group.

° See Table 1 for diet compositions. GG, guar gum; P, pectin; CO, corn oil.

° With adenocarcinoma

<table>
<thead>
<tr>
<th>Diet</th>
<th>n</th>
<th>Mean ± SE</th>
<th>Mean ± SE</th>
<th>Row means</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% fiber</td>
<td>12</td>
<td>50.67 ± 7.5</td>
<td>50.13 ± 7.7</td>
<td>50.36</td>
</tr>
<tr>
<td>5% GG</td>
<td>10</td>
<td>42.57 ± 10.8</td>
<td>42.56 ± 6.5</td>
<td>42.43</td>
</tr>
<tr>
<td>10% GG</td>
<td>10</td>
<td>39.80 ± 6.4</td>
<td>33.73 ± 7.6</td>
<td>36.39</td>
</tr>
<tr>
<td>5% GG/5% P</td>
<td>9</td>
<td>38.00 ± 9.6</td>
<td>39.88 ± 6.6</td>
<td>39.23</td>
</tr>
<tr>
<td>10% P/5% CO</td>
<td>7</td>
<td>22.43 ± 5.5</td>
<td>21.77 ± 4.6</td>
<td>33.89</td>
</tr>
<tr>
<td>10% P/10% CO</td>
<td>10</td>
<td>29.19 ± 7.1</td>
<td>29.06 ± 5.8</td>
<td>29.08</td>
</tr>
<tr>
<td>10% P/20% CO</td>
<td>2</td>
<td>19.00 ± 19.0</td>
<td>25.00 ± 21.0</td>
<td>29.08</td>
</tr>
</tbody>
</table>

Column means 37.50° 37.60°

° See Table 1 for composition. GG, guar gum; P, pectin; CO, corn oil.

° ACF were counted in a 2-cm section of the descending colon from 2.5 to 4.5 cm above the level of the pelvic rim and the full width (1.25 cm) of the colon; total area was 2.5 cm²; \( n = \) number of rats in each category.

° Two-way analysis of variance shows that whether or not a rat has a descending colon tumor (see column means), there was no different in the total number of ACF/rat. However, there was a difference in number of ACF/rat due to the type of diet consumed by the rat (see row means). Specifically, one-way analyses of variance followed by SNK of the row means revealed that the rats that consumed 10% pectin and either 10 or 20% corn oil had significantly \( (P < 0.05) \) fewer ACF/rat than the rats which consumed the 0% fiber diet.
onstrate the influence of a carcinogen on induction of ACF or to identify ACF as putative preneoplastic lesions and have provided valuable information. However, these past studies did not determine if alteration in the number of ACF/rat was significantly correlated with an alteration in the incidence of colon cancer. Two recent reports have raised questions about the ability of number of ACF to accurately predict colon cancer incidence. ACF were observed in animals and their incidence was not positively correlated with the expected alteration in colon cancer incidence. Neither of these two recent studies was able to use the findings of previous reports, e.g. Refs. 17 and 18. In this regard, regression or remodeling of aberrant foci to normal tissue is not a novel idea. For example, researchers have found that 90–98% of aberrant foci in the liver of carcinogen treated rats (19) and a high percentage of hyperplastic or dysplastic epithelial cell foci of the cervix (20) will remodel to phenotypically normal liver or cervical tissue, respectively.

Dietary intervention could have altered the rate of transformation of ACF to adenocarcinoma rather than have altered the absolute number of ACF. The results from the groups of rats which consumed fiber in the diets versus the group of rats which did not consume fiber (0% fiber) in the diet. Statistically, there is also the possibility that if the rate of transformation of ACF to a malignant phenotype is very low, the number of rats in this study may not be large enough to detect a relationship between ACF and colon adenocarcinoma incidence. Given such a very low rate of transformation of ACF to adenocarcinoma, then in an individual the number of ACF is likely to be a poor biomarker for the reduction of colon cancer risk by dietary intervention.

Another alternative possibility is that the original premise that ACF are precursors to colon cancer is incorrect. Therefore, the number of ACF would not be expected to be predictive of adenocarcinoma incidence. Regardless of which of the alternatives is operational, the conclusion remains that when dietary intervention occurred during the promotion phase of colon carcinogenesis, the number of ACF/rat in the descending colon was not a reliable quantitative biomarker for assessment of the efficacy of dietary intervention at reducing the incidence of colon cancer.

This study illustrates the necessity of endpoint validation of potential biomarkers before the predictive value of the biomarker candidate can be properly assessed. Previous investigators have concluded that ACF may be preneoplastic lesions in the descending colon and that induction of ACF may be a good test of the carcinogenicity of a substance. However, the results of this study reveal that the number of ACF as counted in the promotion stage of colon carcinogenesis is not correlated with colon cancer incidence and therefore is not a good single biomarker for the efficacy of a dietary intervention. It therefore seems important that this limitation on the use of number of ACF as a biomarker for colon cancer risk be recognized. If ACF in the descending colon are preneoplastic lesions, it may become necessary to develop additional biomarkers and to use the information about ACF as well as other intermediate biomarkers in order to reliably predict the efficacy of intervention procedures to reduce colon cancer risk in an individual.

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References

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