Recent Hypotheses for the Origin of Colon Cancer

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The origin of many of the cancers seen in Canada, the United States, and other Western countries appears to be associated with diet. International data and migrant studies have shown that cancer of the colon and breast are related to environmental factors (1–4) and international studies suggest that cancers of the colon, breast, pancreas, and prostate are associated with diets high in meat and dietary fat and low in fiber and cereal grains (5–7). However, the specific dietary risk factors responsible for these cancers are still unclear despite considerable effort in epidemiological cohort and case-control studies (8–10). Experimental methods are probably needed to help define the risk factors associated with diet.

The colon is perhaps the best site for carrying out such experimental studies. At this site it is possible to examine the chemistry of the environment in the colonic lumen. It is possible to watch the development of the disease directly with the endoscope. It is possible to biopsy the portion of colon at risk and examine it microscopically. All the steps involved in the development of colon cancer can be observed: the effect of diet on the chemistry in the lumen of the colon; the effect of this chemistry on the cellular biology of the epithelial cells of the colon; the effect of cellular changes on the development of the disease. Hypotheses for the origin of colon cancer can be developed and tested with experimental methods that are much more rapid than the long period of time involved between diet exposure and the onset of cancer.

Here we will review recent experience in the development and testing of five such hypotheses. They have resulted from studies that were initially directed toward the identification of carcinogenic initiators and promoters in the colonic environment (11) and are summarized in Table I. They are being tested for their consistency with laboratory, epidemiological, and clinical studies. The present status of these tests is summarized in Table 2.

Fecapentaenes, Fecal Mutagens of Bacterial Origin

Here we assumed that the colon carcinogens responsible for human cancer arose from within the colonic lumen and are present in feces. We also assumed that these carcinogens were mutagenic and could be detected on that basis. With these assumptions in mind, we examined fractions of feces for their mutagenicity using the Salmonella tester strains (12) and soon discovered that an organic extract of the lipid fraction of many feces contained mutagens that were active on tester strains TA-98 and TA-100 without activation (13). They were associated with a characteristic UV absorption (a “triplet” at 320, 340, 360 nm) and were very sensitive to light, oxygen, and acidic pH (14). It took a much longer time to determine their structures. They were shown to be a group of related ethers of glycerol and unsaturated alcohols containing 5 conjugated double bonds (15–19) and were called fecapentaenes. Fecapentaenes are produced from unknown precursor compounds by certain strains of Bacteroides (20). Their levels are reduced in individuals whose diet is supplemented with ascorbic acid and \( \alpha \)-tocopherol (21) or dietary fiber (22). It remained to be shown that fecapentaenes were implicated in colon cancer causation.

Tests of the association between fecapentaenes and colon cancer have been made in several ways. (a) There have been efforts to assess the carcinogenicity of the fecapentaenes. There appears to be little doubt that these compounds produce genetic change in mammalian cells (23) and will be carcinogenic under certain circumstances (24). However, a problem arises in evaluating the exposure of the colon cells at risk in the animal because of the great instability and reactivity of fecapentaenes. Recently a method for formulating suppositories containing the fecapentaenes in a protected environment at a concentration many thousand times the concentration seen in the human colon has been developed. These suppositories do not produce any evidence of toxicity to the colon over a prolonged period of exposure. (b) The frequencies of fecal mutagens in populations with different incidence of colon cancer have been compared. The frequency is lower in rural black South Africans than it is in urban blacks or whites (25). These results indicate an association between the mutagen and the disease in populations. (c) Two studies of the association of fecal mutagens and colon cancer were made, a small case-control study (26) and an autopsy study (27) of the association of fecal mutagens and colon polyps, hypothesized to be precursors of colon cancer (28, 29). Neither of these studies showed an association between fecal mutagenicity and colon cancer. (d) The results of a recent randomized trial of the effect of diet on colonic polyps bears on the question (30). Two hundred patients who had had a polypectomy and whose colons were free of polyps by colonoscopic examination were randomized into two groups. One group was given capsules containing ascorbic acid and \( \alpha \)-tocopherol (400 mg of each daily) while the other was given placebo capsules. Two years later the patients were reexamined for polyps. There was no statistically significant difference in polyp recurrence between the two groups. Forty % of the patients who received the vitamin supplement and 52% of those receiving the placebo had polyps (31). Thus supplementation of the diet with antioxidants did not appear to have a major effect on this stage of the disease although previous studies had shown that such a supplement reduced the levels of mutagens (21).

I think that, taken together, these tests do not support an important role for the fecapentaenes in colon cancer causation. Although other prokaryotic assays (32–36) may well lead to agents that show a greater association with the disease in time (37), I think it is more efficient to search for genotoxic agents acting on the colon with assays based on mammalian cells in the colon itself.

3-Ketosteroids, Cytotoxic Steroids, and Possibly Genotoxic Steroids in the Fecal Stream

In these studies we assumed that the carcinogens produced signs of toxicity in the colonic epithelium in the form of micronuclei. The assumption was based on the observation that...
carcinogens are frequently clastogenic and induce chromosomal aberrations in the target tissue (38). The chromosomal fragments that are formed are often not attached to the spindle structure and not included in the nuclei of the daughter cells. They give rise instead to micronuclei which are readily scored to provide a rapid, quantitative assessment of the frequency of chromosome aberrations induced in the cell population (39-41). Cells containing micronuclei are usually not viable. Thus the assay is not a true measure of this form of mutation frequency per se but gives an indirect measure of the clastogenic properties of an agent which in many cases corresponds to its carcinogenic potential. When colonic cells were examined after exposure to colon carcinogens, micronuclei were observed but, in addition, a number of other nuclear aberrations or anomalies that include karyorrhexis, pyknosis, and partially digested nuclei were seen in the proliferative compartment (42). This complicated pattern of cell death has been referred to as apoptosis and has been considered to be a form of programmed cell death in the colon and other tissues (43). However, apoptotic figures could readily be quantitated and it was found that graded doses of colon carcinogens yielded increasing numbers of apoptotic figures, while most noncarcinogens and carcinogens that did not act on the colon showed no such increase (44).

The nuclear aberration assay was first used to assess components of human feces as possible colon carcinogens. Lipid as well as aqueous extracts of feces were instilled into the mouse colon and the level of nuclear aberrations was measured 24 h later. Aberrations were observed with fractions of feces extracted with dichloromethane (45). Purification of the active fraction led to the isolation of two ketosteroids, 4-cholesten-3-one and 5α-cholestan-3-one (46). These are two derivatives of cholesterol that have been known to be present in feces (47). They have the ring structure typical for regulatory steroids such as progesterone and testosterone but they have a hydrocarbon chain on position 17 as in cholesterol; they may interfere in regulatory pathways in unexpected ways (48). Presumably these compounds are formed from cholesterol either in the colon or in the preparation or storage of the food.

The importance of the 3-ketosteroids in the origin of human colon cancer is not known. Their carcinogenicity in experimental animals is also not known although some related cholesterol oxidation products have been shown to be carcinogenic on s.c. injection (49). 4-Cholesten-3-one has been found to induce sister chromatid exchanges in the colonic epithelium,2 a genetic effect often associated with carcinogenic compounds. One case control study has reported that the 4-cholesten-3-one is seen at a significantly higher concentration in cases than in controls (50). Nuclear aberrations are readily observed in biopsies of human colons and their frequency appears to be somewhat higher in specimens from patients with colonic polyps or cancers than in controls.3 Thus the risk associated with nuclear aberrations in the human colon may be due to the ketosteroids in the fecal stream although I think it is important to remember that the colonocytes are exposed to a larger environment than that of the fecal stream.

Pyrolysis Products in Food, Carcinogens That Affect the Colonic Mucosa

In the next studies we thus attempted to use the nuclear aberration assay to determine whether there were chemicals in human foods that affected the colon. Mice were fed a number of food items and were then killed 24 h later. The frequency of aberrations in the colon was significantly elevated when the animals ate fried bacon, hamburger, and dried eggs (51). The chemical agents responsible for the elevated levels have not been isolated and identified. However, a number of known carcinogens and mutagens associated with cooked meats (52-54) were assayed for their ability to produce aberrations. These studies showed that many of the food pyrolysis products, including benzo(a)pyrene, 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, and 2-amino-3-methylimidazo[4,5-f]quinoline, induced significant elevations in the frequency of nuclear aberrations in the colons (55, 56) and were thus presumptive colon carcinogens. Recently one of the pyrolysate products has been found to induce colonic tumors in animal models (57).

There is no direct evidence that the food pyrolysis products are a risk factor for human colon cancer, although this seems not unlikely. A direct test of the association of the consumption of pyrolyzed foods and frequency of nuclear aberrations in the human colon could provide such a link. In carrying out such an analysis, consideration would have to be given to other food substances that are known to influence the nuclear aberration score. Butylated hydroxyanisole, caffeic acid (58), and allyl sulfide (59) have been shown to reduce the number of nuclear aberrations in experimental animals exposed to carcinogens. All three are also associated with an inhibition of carcinogenesis under certain conditions (60-62). Perhaps more interestingly, deoxycholic acid, cholic acid, and high fat diets have been shown to markedly increase the numbers of nuclear aberrations induced by carcinogens in experimental animals (63-65) suggesting that these agents could be cocarcinogens or promoters.

I think that the likely interaction of promoters and cocarcinogens with initiators means that we must clarify the nature of the former before we can proceed confidently with the identification of the latter. A bioassay of potential initiating factors, where factors affecting solubility and cell proliferation are not controlled, is too likely to lead to irrelevant findings.

Deficient Dietary Calcium and the Effect of Acidic Lipids on the Colon

Thus we made the assumption that the determinants of human colon cancer were cocarcinogens and promoters and not the ubiquitous initiators. We also assumed that promoters of carcinogenesis may induce colonic cell proliferation (66, 67) and that cell proliferation can be used as an endpoint in looking for such agents. This approach is supported by observations suggesting that the proliferation of epithelial cells of the rectum

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2 H. Kaut et al. manuscript in preparation.

3 W. R. Bruce et al., manuscript in preparation.
is higher in populations at high risk for colon cancer and that the proliferation in normal epithelium of patients with polyps and cancer is higher than that in patients without colon pathology (68–72).

We began our search for promoters among the bile acids that have been known to have this property for many years (73, 74). The toxicity of the bile acids for the colon epithelium was, in fact, first demonstrated in 1964 (75). This report was followed by many colonic perfusion studies carried out in a wide range of experimental animals as well as in humans. These showed that some bile acids, such as deoxycholic acid, as well as certain fatty acids, such as oleic acid, could damage the colon epithelium when their concentration exceeded about 1–3 mM (76–78). Bile acids could induce a proliferative response, presumably a compensatory proliferation, following the damage they produced in the surface epithelium (79). A simple experimental protocol for increasing the exposure of the colon to bile acids by incorporating cholic acid in the diet showed that this intervention increased the number of tumors in animals exposed to colon carcinogens (80). This established that bile acids are promoters.

It has also been known for a long time that high intakes of fat mobilize the bile acids that are required for solubilization and that there may be an association between the consumption of dietary fat and the levels of bile acids in the colonic stream and incidence of colon cancer (81, 82). In general, populations consuming higher amounts of fat have higher levels of bile acids and have a higher risk for colon cancer. However, the differences do not show up clearly in case control studies (83–85). The appropriate risk factor may not be gravimetric concentration of bile acid in the stool, but rather the state of these bile acids and their bioavailability in the stool.

Evidence that the status of the acidic lipids was important and could be affected by calcium came from several short term animal studies. First was a study of the effect of calcium p.o. on the damaging and compensatory proliferation induced by bile acids administered i.r. (86). It was found that calcium salts given p.o. could significantly reduce this toxic effect of bile acids, presumably by precipitating them as their calcium salts, thus reducing concentration of free bile acids in solution in the colon and rectum (87). Second was a study of this phenomenon in the perfused colon (88). It showed that the damage induced by 7 mM deoxycholic acid at a pH of 7.9 could be almost completely inhibited by adding calcium at a concentration of 4 mM to the perfusate. (It could also be inhibited by reducing the pH of the perfusate to 5.9 as will be considered under “High Fecal pH, an Old Hypothesis Reexamined.”) The damage induced by the bile acid corresponded to the concentration of bile acid in solution and not to the total bile acid in the colonic lumen. The third line of evidence involved model experiments following the method described earlier in which animals were given diets containing cholic acid (80). With this model dietary calcium at a concentration of 1% in the diet was found to markedly inhibit colonic crypt cell proliferation compared with that seen when dietary calcium was only 0.1% (89). A fourth line of evidence came from studies of the effect of boluses of fat p.o. on the colonic epithelium and on colonic cell proliferation. Mice were given small p.o. gavages of fat as corn oil or beef tallow (90). Within 2 h there was evidence of surface epithelial loss and by 16–18 h there was a wave of crypt cell proliferation. This effect was not inhibited by administering the fat with a sugar or protein but was inhibited by dietary minerals, starch, and cellulose and also increased dietary calcium (91).

Tests of the importance of dietary calcium to the origin of colon cancer have been made in several ways. Two animal carcinogenesis models have been used to examine the effect of dietary calcium on the promoting effect of fat. Both studies failed to show a protective effect of the calcium as calcium monophosphate at concentrations of between 0.1 and 1% by weight of the diet (92). A further study which examined the effect of changes in both calcium and phosphate also failed to show an effect of calcium on the promotional effect of fat on colon carcinogenesis.6 International ecological data also do not support an important effect of dietary calcium in human cancer (93). Two case-control studies have also failed to demonstrate a protective effect of calcium (94). In contrast, a cohort study has found that dietary calcium was lower in subjects who developed colon cancer than in the control population (95) and a pilot intervention study suggested that supplementary calcium could lead to a reduction in colonic proliferation in individuals judged to be at high risk for colon cancer (96).

It is hard to reconcile the repeated demonstration of the effect of dietary calcium in the animal studies with the lack of effect in animal carcinogenesis studies and with the conflicting human studies. I think it is possible that the levels of calcium intake used in the animal studies are not reached in many of the human populations studied. I think it is also possible that the effects of calcium on proliferation are only transitory. Perhaps there is some form of adaptation.

High Fecal pH, an Old Hypothesis Reexamined

Physicochemical considerations (97–99) gave us a further perspective by suggesting that pH could be the key determinant to the concentration of acidic lipids in the feces. At a high pH, say 9, most of the bile acids in feces are in solution. As the pH is reduced, more of the acids are in their protonated form and drop out of solution so that at a pH of 6 their concentration is very low. As a consequence, there is a marked change in the solubility of the bile acids over the range from 6.5 to 8.0.

Results obtained from model animal systems support an important role of luminal pH on the colonic epithelium. First, as described earlier, the damaging effect of deoxycholic acid on the perfused colon is markedly pH dependent. At a pH of 5.9 there is little effect while at a pH of 7.9 damage can be extensive (88). Second, studies of the proliferation of colons of animals fed low levels of cholic acid in their diets show that proliferation is reduced to control levels in animals whose feces have been acidified. In one of these studies, the pH of the colon was reduced with dietary Na₂SO₄ and also with lactulose,7 and in the other with wheat bran fiber.8 However, another group of studies has reported that high levels of dietary fiber that markedly reduce fecal pH in low fat diets increase epithelial proliferation and increase sensitivity to colon carcinogens (100), an effect similar to that of i.r. acetic acid (101).

However, tests of the association of fecal pH with colon cancer have in general supported a protective effect of a slightly acidic pH. Dietary lactulose and sodium sulfate at levels that reduce pH about 1 unit appeared to have a protective effect in an animal carcinogenesis study (102). Several ecological studies have commented on the higher fecal pH observed in areas with high colon cancer incidence compared with those with low (103–105), and two small case-control studies found that feces

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4 The abbreviation used is: i.r., intrarectal(ly).
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7 R. P. Bird, personal communication.
8 R. P. Bird, personal communication.
9 R. P. Bird, manuscript in preparation.
10 R. P. Bird, manuscript in preparation.
of individuals with colon cancer had higher pH than control (106, 107). There has been no direct test of the effect of dietary manipulation of fecal pH on colonic proliferation although such a test would seem possible. We have found in a short term diet study that subjects receiving diets high in fat and low in fiber have fecal water bile acid concentrations that are higher and more cytotoxic than those of subjects on low fat, high fiber diets (108). We have also recently carried out a controlled blinded study that showed that the proliferation rate in the human colon could be affected by diet (109). In this study the effect of a diet containing 30 g fat per day was compared with diets containing 120 g per day where the difference was made up of 90 g of corn oil as an evening bolus or as three 30-g doses with the meals. After 5 days on the test diets, the proliferation rate as measured by tritiated thymidine labeling of cells from the rectum was approximately 2 times higher in the high fat bolus group. The increased proliferation was associated with a higher concentration of both bile and fatty acids in the fecal water of the feces and interestingly was most marked in older individuals. Thus dietary changes can have effects on the concentration of bile acids and can change the rate of colonic proliferation. Presumably fecal pH was changed in these studies, but unfortunately it was not measured in either one.

The results of an on-going clinical trial will probably bear on the importance of soluble acidic lipids in the colon carcinogenesis process (110). In this study patients who have a “clean” colon following a polypectomy for adenomatous polyps are being randomized into two groups. In the first, the patients eat their normal “Western” diet, while in the other, the diet is modified to provide less than 50 g of fat and more than 40 g of dietary fiber. These patients are being followed for 2 years and are then being reexamined for colonic polyps. Our studies could test the hypothesis that the patients on the low fat diet have a lower colonic pH, a lower concentration of bile acids in their fecal water, and a lower colonic proliferation rate than the control subjects. Thus the importance of these mechanisms on the development of this stage of the disease will be evaluated shortly.

Incidentally, I should add that Metchnicoff (111) suggested in 1908 that fecal acidity might be important in reducing colonic toxicity.

The approach we have used to develop and test hypotheses for the origin of colon cancer has involved two steps: (a) the use of a biological assay to define possible carcinogenic factors; and (b) the test of the importance of these factors. In a, the environment is screened for agents that are active with respect to a laboratory end point and a hypothesis is generated; in b, the hypothesis is tested for its ability to explain the occurrence of the disease.

In looking for possible carcinogens, one must necessarily decide how and where to look. With regard to the “how,” we have used three methods for looking for the carcinogenic factors, a mutational assay and the nuclear aberration assay for possible initiating agents and colonic epithelial cell proliferation for possible promoters. These screens have been effective in that they have led to a number of possible hypotheses. They can hardly be considered to be definitive methods and there is a need for more specific assays for defining carcinogenic factors, assays perhaps closer to the direct testing of environmental mixtures for carcinogenicity (112). The development of an assay based on the quantitation of dysplastic crypts may provide such a screen in the near future. With relation to the “where,” we have used our screens to examine primarily the luminal environment, the fecal chemistry. In the future we should pay attention to the vascular exposure of the colonic cells. Promoting factors associated with dietary fat lead to promotion of breast (113) and pancreatic cancer (114) and whatever factors reach the cells in these organs (115) probably also affect the colonocytes and could have widespread effects throughout the body.

In assessing the importance of the hypotheses for etiological agents, we have used three tests: (a) we have asked whether the isolated and identified agents could induce or promote cancer in animals; (b) we have asked whether the agent was associated with the disease in human populations in ecological, case-control, and cohort studies; (c) we have asked whether the removal of the agent from the environment leads to a reduction of the human disease. These tests are adaptations of tests of causality in disease that have a long history (116). The application of the tests to the five hypotheses we have presented is summarized in Table 2.

I think it is evident on reviewing Table 2 that none of the hypotheses we have developed is fully supported by the application of the tests, yet the colon has fulfilled our expectations as a site for carrying out studies relating diet and cancer risk. We have isolated a number of putative carcinogenic factors from the colonic lumen, we have shown that they are affected by diet, we have shown how they affect the cellular biology of the colonic epithelium, and we are relating these cellular changes to the development of disease. The experimental methods can be carried out in relatively short periods of time and promise to help define the risk factors associated with our diet.

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