Experimental colonic carcinogenesis: changes in faecal bile acids after promotion of intestinal tumours by small bowel resection in the rat

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SUMMARY Small bowel resection promotes the development of colonic tumours in azoxymethane treated rats. As high faecal bile acid concentrations are associated with colonic cancer and may be altered by resection, we have studied changes in faecal bile acid concentrations during promotion of colonic carcinogenesis by increasing small bowel resection. Twenty rats in each group underwent either jejunal transection or 20%, 50%, or 80% proximal small bowel resection. Tumours were induced with azoxymethane 10 mg/kg by 12 weekly subcutaneous injections, and faecal bile acid concentrations were measured at six and 16 weeks. Colonic tumour number rose from 0.6 per rat in the transection group to 1.6 per rat in the 50% resection group ($p < 0.01$) but were not significantly different to transection values at 0.8 per rat in the 80% resection group. Total daily faecal bile acid excretion and bile acid concentrations fell with increasing resection from 14.2 (1.6) mg/rat/day and 5.8 (0.7) mg/g dry faeces respectively in the transection group to 6.5 (0.5) mg/rat/day and 2.9 (0.2) mg/g respectively in the 80% resection group ($p < 0.001$). The greatest reduction was seen in the concentration of deoxycholic acid which has been particularly associated with the aetiology of colonic cancer. The promotion of colonic tumours following small bowel resection in carcinogen treated rats is unlikely to be mediated by changes in faecal bile acid concentration or composition.

Faecal bile acids have been implicated as an aetiological factor in colorectal cancer on both epidemiological and experimental grounds. Marked differences in the incidence of this disease between different populations show an association with the concentration of bile acids in the faeces¹ and high faecal bile acid concentrations have been reported in patients with colorectal cancer and adenomatous polyps.^{2,3} In experimental models of this disease in rats, lithocholic and deoxycholic acid instilled intrarectally have been shown to promote the development of tumours.^{4,5}

Small bowel resection promotes the development of colonic tumours in carcinogen treated rats⁶⁻¹⁰ and this has been attributed to the development of an adaptive hyperplastic response in the colon to the

resection.^{9,11} It is possible that shortening of the bowel may result in increased delivery of bile acids to the colon which may result in tumour promotion. This study was undertaken to compare the changes in faecal bile acid excretion to promotion of tumours in rats treated with the site specific carcinogen, azoxymethane, after small bowel resection.

Methods

ANIMALS

Eighty male Wistar rats weighing between 200 and 240 g were housed in groups of five in animal quarters with a 12 hour day/night cycle and were fed a standard pelleted diet (Labsure CRM, Poole, Dorset) with water *ad libitum*. The diet contained 2.4% fat, 17.9% protein, 57% carbohydrate and 3.6% crude fibre. Animals were randomly allocated to four groups of 20 rats. Group 1 underwent jejunal

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transection and re-anastomosis alone and groups 2 to 4 underwent 20%, 50%, and 80% proximal small resection as measured from the ligament of Treitz to the ileocaecal junction. All operations were carried out under inhalational anaesthesia with 50% O₂/50% N₂O plus 3% halothane, black silk interrupted sutures being used for all anastomoses. Tumours were induced with azoxymethane (Ash Stevens, Detroit, USA), 10 mg/kg weekly for 12 weeks starting one week after surgery. Rats were weighed weekly and results were adjusted by subtracting the starting weight from subsequent weighings for each rat to accentuate differences between the groups. Twenty four hour collections of faeces were made from each cage during the 6th and the 16th week and were freeze dried and weighed before assay for bile acid content. Animals were killed when moribund according to previously defined criteria¹² or at the end of the study at 26 weeks. A full post mortem examination was done and site and size of all tumours were recorded. Tumours with their adjacent bowel were fixed in 10% buffered formalin before preparation for histological examination. Samples of caecum, descending colon, duodenum and terminal ileum were taken for estimation of crypt cell production rate to assess degree of hyperplasia.

BILE ACID ANALYSES

Faecal bile acids were analysed by a gas liquid chromatographic technique.^{13,14} Two faecal samples from each cage of five rats in each group were analysed. Half gram samples of homogenised freeze dried faeces were extracted in 3 ml glacial acetic acid by incubation at 120°C for 30 minutes in a shaking oil bath. At this stage, 900 µg 23-nor-deoxycholic acid was added to the sample to act as an internal calibration marker of loss during the extraction procedure. Six millilitres of toluene was added to precipitate non-organic matter and 3 ml supernatant was evaporated to dryness before further extraction. Bile acid conjugates were hydrolysed in 20% solution of KOH in ethylene glycol at 220°C for 20 minutes and neutral sterols extracted in petroleum ether before acidification with 1.5 ml 6N HCl. Bile acids were then extracted into ether and evaporated to dryness before methylation with diazomethane. The methylated bile acids were reconstituted in 0.5 ml methanol and 1 µl injected into a 3 ft × ¼ inch QF-1 gas chromatography column run at 240°C with a flame ionisation detector. Individual bile acids were identified by comparison of the retention times of the peaks with the retention times of purified standard bile acids run at the beginning and end of each day's run. Quantification of the peaks was done by making solutions of known concentrations of individual purified bile acids and subjecting these to the same

Table 1 *Operative mortality and disease related deaths*

	Control transection	Resection		
		20%	50%	80%
Operative mortality	2	3	2	3
Disease related death	0	0	1	10*
Surviving to 26 weeks	18	17	17	7

*p<0.001, Fisher's Exact test.

extraction procedure before gas chromatographic analysis. From this, calibration lines were drawn for the main bile acids which allowed bile acid concentrations to be read. These concentrations were then corrected for losses by comparison of the height of the internal standard peak with its expected height.

HISTOLOGY

Formalin fixed tissues were embedded in paraffin blocks and multiple 5 µm sections cut through bowel and tumours. Sections were stained with haematoxylin and eosin and tumours classified into benign or malignant according to whether there was invasion through the muscularis mucosa.¹⁵

CRYPT CELL PRODUCTION RATE

Crypt cell production rate (CCPR) was measured by a stathmokinetic technique.^{16,17} On the morning before being killed, rats were injected with vincristine (Oncovin, Eli Lilly, Basingstoke) 1 mg/kg and killed at intervals from 30 to 180 minutes after injection. Samples of bowel were bulk stained by the Feulgen reaction and crypts microdissected. CCPR was derived by linear regression analysis of the accumulation of metaphase arrested cells within the crypts with time.

STATISTICAL ANALYSIS

Tumour numbers were expressed as mean, median and range and statistical comparisons between the different groups made by the Mann-Whitney U Test. Bile acid results were expressed as mean (SE) and Student's unpaired *t* test was used to compare bile acid concentrations.

Table 2 *Dry faecal weight (grams/rat/24 hrs)*

	6 weeks	16 weeks
Transection	2.45 (0.02)	3.17 (0.11)
20%	2.17 (0.08)*	3.12 (0.04)
50%	2.30 (0.01)‡	3.12 (0.49)
80%	2.24 (0.04)†	2.66 (0.15)*

Values expressed as mean (SE); *p<0.05; †p<0.01; ‡p<0.001 v transection group.

Results

Operative mortality was similar between the four groups. In the 80% resected group, however, there was a significantly larger number of animals killed early because of the development of intestinal tumours (Table 1). The rate of weight gain was similar between the four groups between four and 20 weeks but after 20 weeks, weight in the 80% resected group began to fall because of the development of tumours (Fig. 1). There was a small fall in dry faecal weight at six and 16 weeks with resection (Table 2).

Crypt cell production rates (CCPR) in the transection group were 10.5 (1.7) and 7.2 (1.5) cells/crypt/h in the caecum and descending colon respectively and were not significantly changed by resection. In the duodenum, CCPR showed a progressive rise with small bowel resection. In the ileum, a raised CCPR was found in the 80% resection group but this was not significantly different from the transection group (Table 3).

TUMOURS

Jejunal resection resulted in a significant increase in the number of colonic tumours from a mean of 0.67 per rat in the transection group to 1.61 per rat in the 50% resected group ($p<0.01$). In the 80% resection group, however, tumour numbers were not significantly different to the transection group at 0.76 per rat. Tumours also occurred in the small bowel where resection resulted in a steady promotion in the number of tumours from 0.94 per rat in the transec-

Fig. 1 Weight gain adjusted by subtraction of the starting weight of each animal from subsequent weighings.

tion group to 3.59 in the 80% resection group ($p<0.01$) (Table 4). The distribution of tumours is shown in Figure 2. In the colon tumours were found mainly in the descending and transverse colon. In the small bowel, tumours developed predominantly in the first 3 cm of the duodenum and around the anastomosis. Histology confirmed the presence of tumour in all specimens and allowed an accurate classification into adenomas and adenocarcinomas in 92% of specimens. There were no differences in the ratio of benign to malignant tumours between the four groups.

BILE ACID ANALYSIS

Total daily bile acid excretion fell with small bowel resection from 14.2 (1.6) mg/rat/day in the transection group to 6.5 (0.5) mg/rat/day in the 80% resection group at six weeks ($p<0.001$). Similarly, total bile acid concentration fell from 5.8 (0.7) mg/g dry faeces in the transection group to 2.9 (0.2) mg/g in the 80% resection group ($p<0.001$) (Table 5). The relative proportions of constituent bile acids are given in Table 6. Hyodeoxycholic acid was the principle constituent bile acid identified and accounted for between 35 and 38% of total bile acids. Of the other bile acids identified, deoxycholic acid

Table 3 Crypt cell production rates

	Caecum	Descending colon	Duodenum	Ileum
Transection	10.6 (1.7)	7.2 (1.5)	11.5 (1.9)	13.6 (2.6)
20% } Resection	9.7 (3.7)	5.9 (2.5)	15.0 (3.1)	16.0 (2.5)
50% }	10.1 (1.2)	5.0 (1.2)	19.4 (2.6)	14.8 (4.3)
80% }	10.6 (1.2)	6.1 (2.5)	29.1 (1.4)*	31.6 (9.9)

Cells/crypt/h; values expressed as mean (SE); * $p<0.001$.

Table 4 Tumours in each site

	(n)	Colon			Duodenum		
		Median	Mean	Range	Median	Mean	Range
Transected	18	0.5	0.67	0-3	1.0	0.94	0-3
20% Resected	17	1.0	0.88	0-3	2.0	1.65	0-4
50% Resected all	18	1.0	1.61	0-3*	2.0	2.44	0-5†
50% Resected survivors	17	1.0	1.65	0-3*	2.0	2.53	0-5†
80% Resected all	17	1.0	0.76	0-2	3.0	3.59	0-11*
80% Resected survivors	7	1.0	1.00	0-2	7.0	6.00	2-11†

* $p<0.01$; † $p<0.001$ v transected group.

Fig. 2 Tumour distribution. (A = anastomosis).

formed 15%, cholic acid 8%, and lithocholic acid 7%. Table 7 shows the changes in these constituent bile acids with resection. Hyodeoxycholic acid fell in proportion to total bile acid concentrations to 59% of

transection values in the 80% resection group. The greatest change was seen in the concentration of deoxycholic acid which fell to 27% of transection values after 80% resection.

Table 5 Total daily faecal bile acid excretion and concentration in the 4 groups

Resection	Daily bile acid excretion (mg/rat/day)		Bile acid concentration (mg/g dry faeces)	
	6 Weeks	16 Weeks	6 Weeks	16 Weeks
Trans	14.2 (1.6)	16.4 (0.7)	5.8 (0.7)	5.3 (0.4)
20%	7.9 (0.4)†	12.3 (1.0)†	3.6 (0.2)†	3.9 (0.3)†
50%	8.7 (0.8)†	9.9 (1.0)‡	3.8 (0.3)*	3.1 (0.2)†
80%	6.5 (0.5)‡	6.4 (1.1)‡	2.9 (0.2)†	2.3 (0.3)‡

Values expressed as mean (SE). * $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$ v transection group.

Discussion

Epidemiological studies suggest an association between faecal bile acid concentration and the incidence of colon cancer in different populations. In a study of faecal samples from six populations with varying incidence of colorectal cancer, Hill *et al* found a striking relationship to colorectal cancer incidence.^{18,19} Similarly, Crowther *et al*²⁰ and Reddy and Wynder²¹ have reported a relationship between colorectal cancer incidence and faecal bile acid excretion in different subpopulations in Hong Kong and New York respectively. In addition, migrants

Table 6 Relative proportions of constituent bile acids at each collection (per cent)

Bile Acid	6 Weeks	16 Weeks
Lithocholic acid	7.9	4.7
Isoalloxycholeic acid	7.7	13.0
Deoxycholeic acid	15.3	24.0
Chenodeoxycholeic acid	5.0	6.6
Ursodeoxycholeic acid	8.9	10.5
Hyodeoxycholeic acid	38.1	35.3
Cholic acid	8.6	5.7

from low to high risk areas adopt the incidence of colon cancer of the host country. This has been observed in Japanese migrants to Hawaii, which has a particularly high incidence of colorectal cancer²² and Polish migrants to Australia.²³ Similarly, patients with colorectal cancer and adenomatous polyps show a higher faecal bile acid concentration than control patients in some studies^{2,3} although this finding has not been confirmed in others.^{24,25}

Other studies have shown an association between the incidence of colorectal cancer and the consumption of animal protein and fat in different populations.^{26,27} Studies in patients and controls indicate a higher intake of fat and/or meat in patients with the disease.^{28,29} Aries *et al*³⁰ have proposed a hypothesis to bring together the association of a high fat diet, increased faecal bile acid excretion and certain differences in faecal bacterial flora. They suggest that this diet results in higher counts of bacteria capable of degrading bile acids to potentially carcinogenic compounds and will also increase faecal bile acid concentration. Subsequent studies have shown that intestinal bacteria are capable of degrading bile acids into potential carcinogens.³¹ Similarly, a high fat diet has also been shown to increase faecal bile acid concentrations.³²⁻³⁴

More direct evidence for a role for bile acids in colon carcinogenesis comes from the finding of specific bile acid receptors in colorectal cancers in man³⁵ and increased uptake by colonic mucosa of secondary bile acids in patients with adenomatous

polyps.³⁶ Animal models of colorectal cancer have also been used to investigate the role of bile acids in the promotion of neoplasia. Intrarectal instillation of deoxycholeic and lithocholic acid promotes the development of tumours in carcinogen treated rats.^{4,5} Similarly, supplementation of the diet with 0.2% cholic acid has been reported to promote carcinogen induced colorectal cancer in rats.³⁷ Surgical procedures designed to direct the flow of bile into the distal intestine also result in a promotion of neoplasia.^{38,39,40} In a study on the effects of diets high or low in fat and fibre in carcinogen treated rats, Galloway *et al* reported a striking association between bile acid excretion and number of tumours and concluded that a high fibre diet protected against carcinogenesis by dilution of cocarcinogenic bile acids.⁴¹

This study, however, gave results at a variance with the bile acid hypothesis of the aetiology of colorectal cancer. Small bowel resection might be expected to increase the delivery of bile acids to the colon, and therefore may provide a possible mechanism for the previously reported promotional effect of this procedure on colonic carcinogenesis.⁶⁻¹⁰ Our study, however, shows a fall in total daily bile acid excretion to 61% of control values after 50% resection and 45% after 80% resection. Similarly, bile acid concentration in the faeces fell to 65% of the transection value after 50% resection and 50% after 80% resection. Maximal promotion of colonic carcinogenesis was seen after 50% resection, and no simple association can be determined between bile acid concentration or excretion and the promotion of tumours. Analysis of constituent bile acids shows that the greatest reduction occurred in deoxycholeic acid, the bile acid most associated in epidemiological and animal studies with colorectal cancer.

One possible explanation is that initially, small bowel resection may have resulted in a loss of bile acids greater than could be compensated for by increased synthesis. This would result in a depletion in the bile acid pool and reduction in the rate of

Table 7 Principle constituent bile acids in the four groups with percentage change in square brackets from transection group (mg/g dry faeces)

Weeks	Deoxycholeic	Lithocholic	Hyodeoxycholeic	Cholic
Trans 6	0.95 (0.14)	0.49 (0.16)	2.36 (0.14)	0.53 (0.46)
16	1.42 (0.11)	0.28 (0.01)	2.09 (0.12)	0.34 (0.03)
20% 6	0.46 (0.015) [48]†	0.16 (0.005) [33]	1.59 (0.13) [67]‡	0.52 (0.065) [98]
16	0.74 (0.08) [52]‡	0.51 (0.20) [182]	1.69 (0.17) [81]	0.31 (0.04) [91]
50% 6	0.37 (0.043) [39]†	0.17 (0.016) [35]	1.70 (0.10) [72]†	0.58 (0.095) [109]
16	0.46 (0.021) [32]‡	0.29 (0.07) [104]	1.29 (0.14) [62]‡	0.38 (0.046) [112]
80% 6	0.26 (0.03) [27]‡	0.08 (0.008) [16]*	1.40 (0.13) [59]‡	0.54 (0.058) [102]
16	0.34 (0.053) [24]‡	0.16 (0.010) [57]‡	1.24 (0.17) [59]†	0.28 (0.012) [82]

Values expressed as mean (SE); * $p < 0.05$; † $p < 0.01$; ‡ $p < 0.001$ v transection group.

secretion into the intestine with consequent reduction in faecal excretion. There is some evidence from previous reports that this may be the case. In man, ileal resection of greater than 100 cm resulted in much greater wastage of bile acids than lesser terminal ileal resection.⁴² In patients undergoing distal small bowel resection with preservation of 35–50 cm of terminal ileum, however, bile acid excretion was normal suggesting that only a small segment of terminal ileum may be necessary for efficient reabsorption of bile acids.⁴³ In this study, 15 cm of terminal ileum was preserved in the 80% resection group and smaller resections were proximal to the ileum. It seems unlikely that reduction in bile acid excretion with resection was caused by this mechanism. In this study, resection resulted in an increase in crypt cell kinetics in the small bowel. A previous report has shown that ileal adaptation to jejunal resection results in an increase in bile acid reabsorption and this may account for the fall in faecal bile acid excretion.⁴⁴

In the transection group, the finding of total bile acid concentrations of 5.8 and 5.2 mg/g dry faeces compares well with previous reports. Galloway *et al.*⁴¹ reported 11.9 and 9.4 mg/g in rats treated with or without azoxymethane fed a high fat low fibre diet and 2.8 and 3.8 mg/g in similar groups fed a low fat high fibre diet. The diet used in this study is comparable to Galloway's low fat low fibre diet which resulted in a faecal bile acid concentration of 7.18 mg/g. The analysis of individual bile acids in this study shows considerable concordance with previous reports which show hyodeoxycholic acid to be the principle constituent bile acid in rat faeces.^{41,45} Relative proportions of deoxycholic acid are more variable ranging from 6%⁴³ to 33%^{41,46} these results being in accordance with the results presented here.

In this study, faecal bile acids were measured at six weeks as this was the midpoint of carcinogen administration and therefore could be considered to be during the initiation phase of carcinogenesis. The second collection was made at 16 weeks, one month after the last carcinogen injection and during the period of promotion of tumours by small bowel resection but before the animals showed clinical evidence of disease. The results are similar between the two collections suggesting that azoxymethane at this dose regime does not affect faecal bile acid excretion.

It is of interest to note that small bowel tumours occurred mainly around the duodenal papilla. High concentrations of carcinogen in the duodenum, however, are unlikely to be the cause of the high tumour yield in this site as it has been shown that diversion of pancreatobiliary secretions do not affect the distribution of small bowel tumours after carcinogen

administration.^{38,39} In addition, it is now thought that azoxymethane reaches its site of action by the blood stream^{47,48} rather than by excretion in bile as was previously thought.⁴⁹ We have shown a significant increase in crypt cell kinetics in the duodenum with small bowel resection which is associated with a promotion of neoplasia in this site and supports the association between hyperplasia and neoplasia. In the colon, we could demonstrate no changes in the rate of cell proliferation with resection. Crypt cell production rate is a less sensitive indicator of hyperplasia in the colon, however, as cell turnover is slower and significant changes occurring earlier may not have been detected. Other reports have shown hyperplasia by this technique in the colon of carcinogen treated rats undergoing small bowel resection or bypass.⁹ The promotion of colonic tumours may therefore have been associated with a mild hyperplastic response to resection in the colon.

It is important to determine which factor or factors are responsible for the promotion of colorectal cancer in rats after small bowel resection as this may have implications in patients undergoing bowel resections for non-malignant disease. There are, however, as yet no studies showing an increase incidence of colorectal cancer in patients undergoing this type of resection.

In summary, we have shown that promotion of colonic neoplasia by small bowel resection is associated with a fall in faecal bile acid excretion and concentration. While there is strong evidence that faecal bile acids are important in the epidemiology of colorectal cancer in man and have been shown to promote colonic carcinogenesis in carcinogen treated rats, alteration of tumour induction by small bowel resection is unlikely to be due to changes in total or constituent faecal bile acid excretion or concentration.

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