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• COLORECTAL CANCER •

Ethylene diamine tetraacetic acid induced colonic crypt cell hyperproliferation in rats

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Abstract

AIM: To investigate the effect of ethylene diamine tetraacetic acid (EDTA) on proliferation of rat colonic cells.

METHODS: EDTA was administered into Wistar rats, carcinogenesis induced by 1,2-dimethylhydrazine (DMH) in rats was studied with immunohistochemistry.

RESULTS: Marked regional differences in cell proliferation were found in all groups. In EDTA-treated animals, total labelling indexes in both proximal (10.00±0.44 *vs* 7.20±0.45) and distal (11.05±0.45 *vs* 8.65±0.34) colon and proliferative zone size (21.67±1.13 *vs* 16.75±1.45, 27.73±1.46 *vs* 21.74±1.07) were significantly higher than that in normal controls ($P<0.05$) and lower than that in DMH group (10.00±0.44 *vs* 11.54±0.45, 11.05±0.45 *vs* 13.13±0.46, 21.67±1.13 *vs* 35.52±1.58, 27.73±1.46 *vs* 39.61±1.32, $P<0.05$). Cumulative frequency distributions showed a shift of the EDTA distal curve to the right ($P<0.05$) while the EDTA proximal curve did not change compared to normal controls. Despite the changes of proliferative parameters, tumours did not develop in EDTA treated animals.

CONCLUSION: Hyperproliferation appears to be more easily induced by EDTA in distal colon than in proximal colon. Hyperproliferation may need to exceed a threshold to develop colonic tumours. EDTA may work as a co-factor in colonic tumorigenesis.

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INTRODUCTION

Colonic epithelial hyperproliferation has been considered as a high risk factor in both human and animal colonic cancer models[1-19]. Evidence from animal studies has shown that experimental colonic tumours induced by procarcinogen 1, 2-dimethylhydrazine (DMH) are of epithelial origin with a similar histology, morphology and anatomy to human colonic neoplasms[2,3,20,21]. Furthermore, prior to the development of colonic cancer, injections of DMH could result in increased colonic crypt cellularity, colonic crypt cell proliferation and colonic crypt proliferative zone[22,23]. This procarcinogen thus provides an adequate model for kinetic and therapeutic studies of the colorectal cancer.

It is important to compare cell proliferation in the distal and proximal colon. As in normal rats, the location of stem cells and the direction of colonocyte migration differ in these two regions[24]. In addition, differences in the incidence, morphology and clinical behaviour of colonic carcinoma in the proximal and distal colon have been reported[25,26].

EDTA is widely used as a vehicle solution in chemical-induced colorectal carcinogenesis. However, little is known about the nature of its effect on cell proliferation. *In vitro*, EDTA could inhibit cell proliferation[27-29] and DNA synthesis[30]. Inhibition on cell growth is not associated with EDTA' s chelational stability[28]. *In vivo*, EDTA has been shown to stimulate cell proliferation in a neural crest tumour model[31]. In the present study BrdUrd *in vivo* cell labelling was employed to determine crypt cell proliferation patterns in proximal and distal rat colons from normal, EDTA and DMH-induced colon cancer animals.

MATERIALS AND METHODS

Animals and treatment

Forty eight male Wistar rats (weighing 180-220 g) were divided equally into DMH group and EDTA group. Three animals were housed in each cage in a containment isolator with negative pressure to protect experimenters against the effects of the carcinogen. A specific colonic procarcinogen 1, 2-dimethylhydrazine (DMH, Aldric, Poole, Dorset) at a dosage of 20 mg/kg body weight was administered subcutaneously to the animals weekly for 20 weeks. DMH was dissolved in 1 mM EDTA (BDH Ltd, Poole, Dorset), and adjusted to pH 6.5 with 10% sodium hydroxide (BDH Ltd, Poole, Dorset) immediately before injection. Animals in EDTA group were given weekly subcutaneous injection of EDTA for 20 weeks, and sacrificed 2 weeks after the last injection. In addition, six normal rats were used as controls for BrdUrd immunohistochemistry.

In vivo BrdUrd labelling and tissue sampling

Eighteen rats (6 per group) from normal controls, EDTA-treated group and DMH-treated group were used for a crypt cell proliferation study. Fifteen minutes before removal of the colon, the anaesthetised animals had a peritoneal injection with 50 mg/kg body weight of 2% BrdUrd (Sigma B-5002) between 9 and 11 a.m. to avoid diurnal variation. The colon was removed and rinsed with tap water. Following excision of the caecum and rectum, the remaining colon was divided into proximal and distal halves. A 1-2 cm segment of each end of the proximal and distal colon was discarded. After fixation in 70% ethanol for 4 hours the segments were rolled prior to processing and embedding in paraffin wax.

BrdUrd immunohistochemistry

Several 3 μm thick sections were cut and placed on poly-L-

lysine coated slides. The slides were dewaxed before DNA was denatured in 1M HCl at 37 ℃ for 12 minutes. After rinsed in phosphate buffered saline (PBS, pH 7.1) the sections were incubated with 30 μl of mouse anti-BrdUrd monoclonal antibody (M 744 Dako, Bucks, England) diluted 1:50 in PBS with 0.05% Tween 20 (PBST) with added normal rat serum diluted 1:25 for 60 minutes at room temperature. After a further rinsing in PBS the sections were incubated with biotinylated rabbit anti-mouse F(ab')$_2$ antibody (E 413 Dako, Bucks, England) at a dilution of 1:200 in PBST with added rat serum for 30 minutes at room temperature. The slides were again rinsed in PBS and then incubated with streptavidin-biotin peroxidase complex (K 377 Dako, Bucks, England) for 30 minutes at room temperature. Finally the reaction product was visualised using diaminobenzidine hydrochloride (DAB) (Sigma, Dorset, England) primed with 100 μl of 30% H_2O_2 (diluted 1:20 with distilled water) for approximately 5 minutes. After DAB was washed off with distilled water the sections were lightly counterstained in Harris haematoxylin before dehydration and mounting in DPX.

Counting and scoring criteria

Only complete well-orientated longitudinally sectioned crypts which extended from the luminal surface into the muscularis mucosae and contained at least 30 cells per hemicrypt were used for analysis. To facilitate scoring each crypt was divided at the base into 2 crypt columns (hemicrypts). Starting at the base of the hemicrypt, cells were numbered up to the luminal surface of the colon to determine the number of cells per hemicrypt. Crypts were then divided into 5 compartments each containing the same number of cells. The number and the position of BrdUrd-labelled cells in the hemicrypt were recorded. The proliferative zone, which was expressed as a percentage, was obtained by calculating the difference between the highest and lowest labelled cells in each hemicrypt and dividing this figure by the total number of cells in the hemicrypt. Labelling index (LI) was determined for the whole hemicrypt, for each compartment and for the proliferative zone as follows, equation=(the number of labelled cells/the number of total cells)×100. Each hemicrypt was then normalised to a notional 100 cell positions. The frequency of BrdUrd positive cells in each of the 100 normalised positions was recorded.

Statistical analysis

Mean and standard error of the mean were calculated where appropriate. Since the sample size for crypt cell proliferation was more than 100 except in one group (normal proximal colon group in which the sample size was 78) and all groups appeared to have normal distribution, a two sided Student's *t* test was used to identify the differences between individual variables. Kolmogorov-Smirnov 2 sample test[16] was used to compare the BrdUrd cumulative labelling frequency curves with reference to the presence of EDTA or DMH treatment and the site of origin of the sample from the colon. Results were considered as significant when $P<0.05$. Statistics were analysed running the SPSS package for Windows.

RESULTS

Characterisation of tumours

All animals survived to the time when they were sacrificed. In the EDTA control animals no macroscopical changes were observed in the mucosa of large intestine after 20 weeks of treatment. However, in the DMH group a total of 66 tumours were found in 23 animals (96%) and 1 rat was tumour free. Most of the tumours (73%) were located in the distal colon. Microscopically, the tumours were either adenoma or adenocarcinoma. Most rats had one or more types of lesions, indicating that the response to DMH was heterogeneous. The number and distribution of both adenoma and adenocarcinoma are summarised in Table 1.

Table 1 Characterisation of colorectal tumours

Location	Adenoma	Carcinoma	Total (%)
Caecum	0	1	1 (1.5)
Proximal	2	6	8 (12.1)
Flexure	4	4	8 (12.1)
Distal	12	36	48 (72.7)
Rectum	0	1	1 (1.5)
Total	18	48	66

Differences between proximal and distal colon

The number of cells per hemicrypt in the proximal colon was significantly higher than that in the distal colon in all respective groups ($P<0.05$, Table 2). Although the number of labelled cells per hemicrypt was similar in the proximal and distal colon in all groups, the total LI was significantly higher in distal colon compared to proximal colon both in normal control group ($P<0.05$) and in DMH treated animals ($P<0.05$). In EDTA treated group, the total LI in the distal colon was higher than that in the proximal colon with no statistical significance ($P=0.11$). The size of the proliferative zone was higher in distal colon than that in proximal colon in all groups ($P<0.05$) while the LI in proliferative zone did not significantly change. In the normal controls, BrdUrd labelled cells in the proximal colon were located predominantly in compartments 2 and 3 (88.4%), whereas the labelled cells in the distal colon were mostly in compartments 1 and 2 (85.3%). In compartment 1, the LI was significantly higher in the distal colon than in the proximal colon ($P<0.05$), while in compartment 3 the LI was significantly lower in the distal colon than in the proximal colon ($P<0.05$). None of the labelled cells appeared in compartment 5. When the cumulative labelling distribution curves of the proximal and distal colon of normal rats were compared, the distal colon showed a significant shift to the left ($P<0.05$, Figure 1).

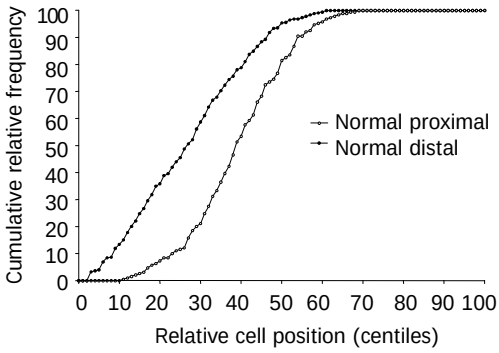

Figure 1 Different patterns of cumulative labelling distributions in proximal and distal rat colons of normal controls. The curve was significantly shifted to the right when the proximal colon was compared to the distal colon.

Effect of EDTA

In EDTA-treated animals, the number of cells per hemicrypt, labelled cells per hemicrypt and total LI were all significantly increased in both the proximal and distal colons when compared to normal controls. In addition, the size of proliferative zone in EDTA treated animals was significantly higher in both proximal and distal colons than in normal controls. However, the LI in proliferative zone was not changed. In the proximal colon, the increase in LI was limited

to compartment 3 while in the distal colon the increase in LI was extended from compartment 2 to compartment 4. When compared to the normal controls cumulative frequency distributions of the EDTA distal curve shifted to the right (P<0.05, Figure 2) but the EDTA proximal curve did not change (P>0.05, Figure 3).

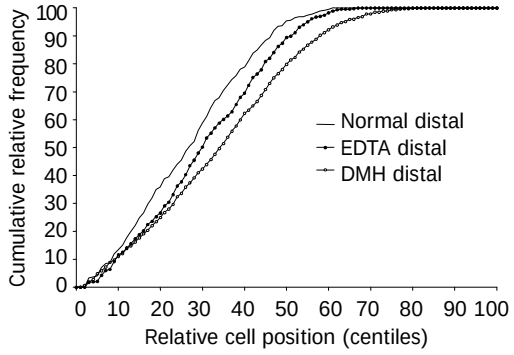

Figure 2 Cumulative labelling distribution in normal, EDTA and DMH treated distal rat colons, respectively. The curve was significantly shifted to the right in EDTA distal colon compared to normal distal colons. The curve was further shifted to the right in DMH distal colon compared to either normal or EDTA distal colon.

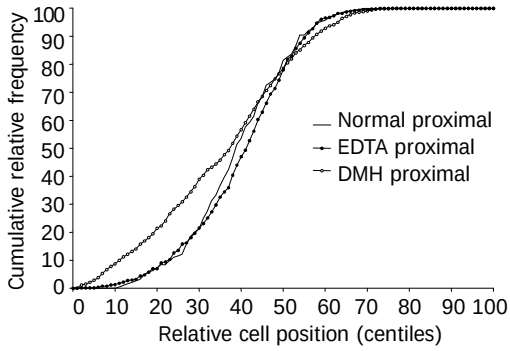

Figure 3 Cumulative labelling distribution in normal, EDTA and DMH treated proximal rat colons. The curve of EDTA treated proximal rat colon did not change compared to the normal proximal curve. Whereas the curve of DMH treated proximal rat colon was initially significantly shifted to the left and then at higher centiles shifted to the right.

Effect of DMH treatment

DMH treatment significantly increased the number of cells per hemicrypt in both proximal and distal colons in comparison with either normal controls or EDTA treated animals. The number of labelled cells per hemicrypt and total LI were also significantly increased following DMH injections in both proximal and distal colons. Additionally, DMH increased the size of the proliferative zone in both proximal and distal colonic crypts. However, the LI of proliferative zone was reduced because of the increased denominator (Table 2). In the proximal colon the increase in LI was in compartments 1, 3 and 4, whereas in the distal colon the increase in LI in DMH rats was most marked in compartments 2, 3 and 4. The extent of LI in compartment 3 in the distal colon was significantly greater than that in the proximal colon (P<0.05). Further analysis of the cumulative labelling distributions showed a shift of the DMH distal curve to the 81st centile which was to the right of the plateau of the normal distal colon located at the 61st centile (P<0.05) and EDTA distal colon located at the 67th centile (P<0.05, Figure 2). In contrast, the cumulative labelling distribution curve in DMH proximal colon demonstrated a shift to the left in the lower crypt cell positions and then shifted to the right high up the crypt compared with normal (P<0.05) and EDTA (P<0.05) proximal cumulative frequency curves (Figure 3).

DISCUSSION

This study supported the findings that adenoma and adenocarcinoma of intestine could be obtained, using DMH or its metabolites in Wistar rats[20,32,33]. Another advantage of DMH and its metabolites is the specificity for the large bowel. The incidences of cancer at small intestine and extra-intestine were very low[34]. In our study the vast majority of colon tumours occurred distally and were mainly polypoid neoplasms or adenocarcinomas. The types of the proximal colon tumours were reported to be variable histopathologically, dominated

Table 2 Features of proliferative colonic crypt cells in 6 groups

Group	LC	CPC	TLI	LI 1	LI 2	LI 3	LI 4	*P* Zone	LIPZ
NP(76)	2.49±0.16	34.45±0.39	7.20±0.45	3.11±0.11	18.78±1.64	13.21±1.50	0.90±0.39	16.75±1.45	59.43±3.39
ND(198)	2.74±0.11	31.45±0.2	8.65±0.34	17.33±1.06	19.54±1.21	6.38±0.76	0.00	21.74±1.07	55.13±2.17
EP(134)	3.71±0.16	37.68±0.27	10.00±0.44	3.36±0.62	19.46±1.41	24.45±1.55	1.67±0.43	21.67±1.13	55.87±2.22
ED(130)	3.58±0.15	32.55±0.21	11.05±0.47	14.76±1.33	23.74±1.66	15.49±1.29	0.9±0.31	27.73±1.46	51.68±2.43
DP(138)	5.7±0.24	49.09±0.65	11.54±0.45	12.87±1.11	21.72±1.41	19.72±1.21	3.47±0.62	35.52±1.58	39.84±1.77
DD(182)	5.67±0.23	42.68±0.53	13.13±0.46	18.23±1.08	24.86±1.31	18.83±1.19	3.43±0.6	39.61±1.32	38.89±1.50
P Values									
ND:NP	0.21	0.000	0.019	0.000	0.731	0.000	0.000	0.011	0.3
ED:EP	0.571	0.000	0.107	0.000	0.05	0.000	0.151	0.001	0.203
DD:DP	0.94	0.000	0.017	0.003	0.107	0.604	0.966	0.047	0.682
ND:ED	0.000	0.000	0.000	0.131	0.037	0.000	0.000	0.001	0.3
ND:DD	0.000	0.000	0.000	0.556	0.003	0.000	0.000	0.000	0.000
ED:DD	0.000	0.000	0.002	0.043	0.593	0.062	0.001	0.000	0.000
NP:EP	0.000	0.000	0.000	0.798	0.765	0.000	0.237	0.009	0.365
NP:DP	0.000	0.000	0.000	0.000	0.195	0.001	0.004	0.000	0.000
EP:DP	0.000	0.000	0.016	0.000	0.259	0.017	0.019	0.000	0.000

Values represented as $\bar{x}\pm S_x$, NP(76): Normal proximal, 76 hemi-crypts were counted, ND: Normal distal, EP: EDTA proximal, ED-EDTA distal, DP: DMH proximal, DD: DMH distal, LC: Labelled cells per hemi-crypt, CPC: Cells per hemi-crypt, TLI: Total labelling index, LI1: Labelling index of compartment 1, Pzone: Proliferative zone size, LIPZ: Labelling index of proliferative zone Size.

with the mucinous type of adenocarcinoma[35]. The different responses of bowel segments to DMH, and the reasons for the predilection of colonic neoplasia to distal colon as well as the differences in morphological type of tumours between the proximal and distal colon are not fully understood. However it is known that the structure and function of intestinal mucosa differed significantly between humans[36] and experimental animals[37-39]. An understanding of these inherent regional differences may be pivotal in study of the mechanisms of colonic tumorigenesis.

Significant regional differences in the distribution of BrdUrd-labelled cells in proximal and distal rat colon were demonstrated in this study. The differences in distribution of proliferative cells between proximal and distal colons were previously shown with ^{3}H-thymidine autoradiography and immunohistochemistry. ^{3}H-thymidine LI and proliferative zone size were reported to be significantly greater distally than proximally[40]. In the distal colon PCNA expression was strictly confined to the lower third of the crypt, whereas in the proximal colon it was located in the mid-crypt[23].

Sunter noted that the peak LI in the proximal colon was located in the middle third of the crypt while the peak of LI in the distal colon was located in the lower third near the base of the crypt[41]. These findings, together with ours, tended to support the theory of crypt cell origin and colonocyte migration given by Sato and Ahnen[24]. After a double labelling with ^{3}H-thymidine and BrdUrd, Sato and Ahnen investigated the location of stem cells and the direction of colonocyte migration in normal rat colonic crypt, and reported that distal stem cells were located in the crypt base while proximal stem cells in the mid-crypt, thus postulating that colonocytes migrated up toward the luminal surface in the distal colon in contrast to the bidirectional migration, i.e. up toward the luminal surface and down toward the crypt base in the proximal colon.

Our results showed that after EDTA treatment, the number of proliferative colonic crypt cells was significantly increased in the distal and proximal colon. The LI in the proliferative zone in the EDTA animals did not increase which could be attributable to the concomitant increase of the zone size. In the EDTA treated animals, LIs in all compartments in the distal colon increased except the LI in compartment 1, a significant increase of LI in the proximal colon was found in compartment 3 only. This was corroborated by the cumulative labelling distribution curves. In comparison with normal control, the distal curve shifted toward to the right, but the proximal curve did not change. The fact that the proximal curve did not shift might be important. If hyperproliferation preceded tumour formation and was a cause of tumor formation, then these findings indicated that the distal colon was more susceptible. EDTA did not induce colorectal tumours after administration for 20 weeks, but prolonged EDTA treatment might induce tumours. Another hypothesis is that hyperproliferation in EDTA animals is a co-factor in DMH-induced colonic tumour formation.

DMH treatment further increased colonic crypt cell proliferation. Although DMH treatment increased the LI in both the proximal and distal colon, the cumulative labelling distribution was markedly shifted to the right in the distal colon whereas the proximal curve shifted to the left (*i*.e. downwards in the crypt). The distribution of DMH-induced colorectal cancer resembled human colorectal carcinoma[42-44]. We found that when the total colon was exposed to the procarcinogen DMH, 73% of tumours occurred distally and only 12% proximally.

Further investigation is required to understand the differences of tumour distribution, and their relationship to the proliferation of different crypt cells and differentiation patterns in the proximal and distal colon. It has been shown that in the proximal colon, mucous cells were predominant in the lower third of the crypt, whereas columnar cells in the upper third[45]. In contrast, crypts of the distal colon contained only a small number of mucous cells in basal positions. The undifferentiated cells or the cells with the lowest level of differentiation (presumptive stem cells) were the vacuolated cells located near or at the base of crypt[46]. The progeny of the vacuolated cells migrated upward to differentiate into columnar cells and downward to differentiate into mucous cells[47].

In this study we observed a great number of cells in the crypt in the proximal colon than in the distal colon, which was in contrary to published data[39,40]. This disparity might be due to different criteria for recoding the overlapping nuclei, selecting crypts or ascertaining the top of the crypt. In this study longitudinally well-oriented crypts were selected and all visible nuclei were counted.

EDTA increased crypt cell proliferation but all of the increased parameters were significantly lower than those in the DMH group. The number of crypt cells in hyperproliferation induced by a carcinogen might reach or exceed a limited value (threshold) before colonic tumours developed. While proliferative crypt cells that did not exceed this threshold as in the EDTA animals, might act as a promoting agent to stimulate tumour growth. In conclusion, EDTA' s effect of increasing crypt cell proliferation may be a co-factor in this model.

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