Enhancement of ethanol induced rectal mucosal hyper regeneration with age in F344 rats

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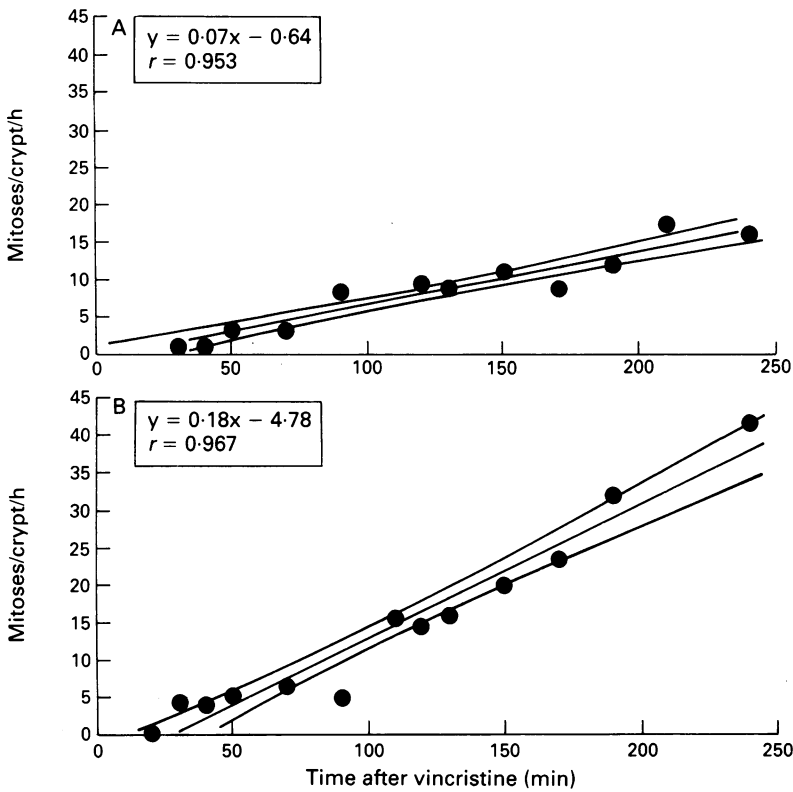
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Abstract
Experimental studies in rats have shown an independent stimulation of rectal cell turnover by either chronic ethanol consumption or age. In this study the combined effect of these two factors on colorectal cell regeneration has been investigated. Ninety male F344 rats aged 2, 12, and 22 months were pair fed nutritionally adequate liquid diets containing 36% of total energy either as ethanol or isoenergetic carbohydrates. After four weeks of feeding, colorectal crypt cell production rates were measured using a stathmokinetic technique with vincristine. While age by itself did not affect colorectal cell renewal, chronic ethanol consumption stimulated rectal, but not colonic crypt cell production rate in an age dependent manner. While no significant effect of ethanol was noted in young animals, cell proliferation was significantly enhanced in middle aged animals by 81% (4·1 (2·7–5·5) v 7·4 (6,0–8·7) cells/crypt/hour, p<0·001) and in old animals by 138% (4·5 (3·3–5·6) v 10·7 (8·9–12·4) cells/crypt/hour, p<0·001) after ethanol ingestion. Because acetaldehyde, the first and most toxic metabolite of ethanol, has been detected in the colorectal mucosa and may lead to tissue injury influencing cell regeneration, acetaldehyde concentrations have been measured in the colons of 15 male F344 rats of various ages after an acute intraperitoneal dose of ethanol (2·5 g/kg bodyweight). There was a significant positive correlation between crypt cell production rate and acetaldehyde concentrations measured in the distal and proximal colon after an acute dose of ethanol ($r = 0.5955$, p<0·005). These data clearly show that the ethanol mediated stimulation of cell regeneration in the rectum is age dependent. As reported earlier, there was found indirect evidence that acetaldehyde participates in the pathogenesis of rectal hyperregeneration after chronic alcohol consumption. This hyperregeneration of the rectal mucosa after alcohol drinking could by itself favour carcinogenesis, which is especially relevant in old age.

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Colorectal cancer is a leading cause of death in industrial countries.[1][2] Studies in experimental animals[3][4] and in humans[5][6] have clearly shown

that regardless of its aetiology epithelial hyperregeneration is predisposing to the development of colorectal cancer. In addition, epidemiological studies have identified both chronic ethanol consumption[7–10] and advanced age[11] as independent risk factors for rectal cancer. Both entities are associated with increased rectal cell turnover in experimental animals.[12–14] Furthermore, labelling studies with tritiated thymidine using human rectal biopsy specimens also show that advanced age correlates with increased rectal cell regeneration.[15] On the basis of these findings we have investigated in this study the combined effect of aging and chronic ethanol consumption on rectal cell proliferation. We used an established animal model in which ethanol was fed as a liquid diet and a stathmokinetic technique with vincristine to measure cell turnover.

Methods

ANIMALS AND DIETS
Ninety male F344 rats aged 2, 12, and 22 months were randomly paired and individually housed in stainless steel wire cages. Such animals are expected to live between 26 and 31 months when fed ad libitum. They were pathogen free and viral antibody free, and came from the Sprague Dawley Company, Indianapolis, Indiana. The animals were maintained in an air conditioned room in single cages (suspended) at a temperature of 23°C and a humidity of 45%. The light cycle was 12 hours. Thirty six per cent of energy was given either as ethanol or isoenergetic carbohydrates as described by Lieber and DeCarli.[16] Protein (18% of calories) and lipid (35% of calories) content were the same, but the diets contained different amounts of carbohydrates (11% in the alcohol fed rats and 47% in the control animals). The ethanol concentration of the diet was 6·2% (vol/vol). Ethanol was introduced into the diet gradually, reaching maximal concentrations after five days. Liquid diets were renewed daily at 10 am to ensure continuous ethanol consumption. The feeding period was four weeks.

MEASUREMENT OF CELL REGENERATION
To measure colorectal cell regeneration, crypt cell production rate (CCPR) was determined in the colons from 13 ethanol fed and 13 control animals of each age group using the metaphase arrest technique.[17] Animals were injected intraperitoneally with 0·8 mg vincristine/kg of body weight, and after 30

Figure 1: Metaphase accumulation after vincristine induced (0·8 mg/kg body weight) metaphase arrest in the distal colons of old (22 month old) control (A) and ethanol fed (B) male F344 rats and their 95% confidence limits. Each data point represents a mean of findings in 10–20 crypts in each animal. Metaphase arrest lines were fitted by least square linear regression analysis. The slope of the line gives the crypt cell production rate expressed as cells/crypt/hour. Chronic ethanol consumption increased the crypt cell production rate significantly (p<0·001).

per circumference of 20 cross sectioned crypts per animal.

ACETALDEHYDE DETERMINATION IN THE COLORECTAL MUCOSA

To measure if aging affects mucosal acetaldehyde concentrations in the colon after an acute dose of ethanol, 15 male F344 rats 2, 12, and 22 months of age received a single intraperitoneal dose of ethanol (2·5 g kg body weight; 30% vol/vol), and 165 minutes thereafter the rats were anesthetised with phenobarbital. The colon was cleared of faeces by in vivo perfusion of the large intestine with ice cold 0·15 M NaCl solution. This procedure took about 10 seconds. Within five seconds after colonic lavage the first 5 cm of the colon distal to the ileocaecal valve (right side of the colon) and the last 5 cm proximal to the anus (left side of the colon) were placed into 4 ml of 2-diphenylacetyl-1,3-indandione-1-hydrazone (DIH) reagent followed by an immediate homogenisation.[13][19]

Acetaldehyde in the colon was determined by high performance liquid chromatography after adduct formation with DIH.[13][19] The original method was modified for tissue analysis as follows: DIH (30 mg/100 ml) was dissolved in acetonitrile-methanol (60:40 vol/vol), and about 1 g of tissue was homogenised in 4 ml of reagent. After centrifugation and development of fluorescence, 1 ml water was added to samples, standards, and blanks before chromatographic separation. The working standard was increased 10-fold to a final concentration of 30 μM.

minutes, pairs of rats were killed at 10 to 30 minute intervals. Tissue samples were taken from the colon (3 cm distal to the ileocaecal valve) and rectum, fixed in Carnoy's solution, dehydrated, and stained by the Fuelgen reaction. Crypt squashes were then prepared by microdissection. Metaphase figures were counted in 10–20 crypts/site, and the number was plotted against time. Metaphase arrest lines were fitted by least square linear regression. The slope of the lines gives the CCPR expressed as cells/crypt/hour.

For estimation of the size of the proliferative compartment a graphical method was used, determing the 50% peak value of the mitotic index distribution curve.[18] Crypt sizes were analysed by counting cells per crypt column of 50 longitudinally sectioned crypts – that is, 100 crypt columns – per animal and cells

STATISTICS

All data were expressed as mean (SD) Statistical comparison of data were performed using the Student's t test. A p value lower or equal to 0·05 was considered significant. In addition, metaphase accumulation data were subject to linear regression analysis by the method of least squares.

Results

Chronic ethanol consumption results in a 35% enhanced cell regeneration in 2 month old rats which, however, did not reach a value of statistical significance (p>0·05). When older animals were studied, the effect of alcohol was strikingly enhanced. Twelve month old F344 rats exhibited a highly significant 81% increase in cell turnover after ethanol consumption (p<0·001). This stimulating effect of alcohol was further increased in 22 month old F344 rats. Figure 1 shows mucosal cell regeneration in the rectum measured as CCPR in these animals. Table I summarises the effect of chronic ethanol consumption and age on CCPR, birth rates, and on the proliferative compartment of the crypt in the distal colon of F344 rats. When CCPR is transformed to and corrected for crypt sizes expressed as cells/1000 cells/hour (birth rate), the combined stimulating effect of chronic ethanol ingestion

TABLE I   Effect of age and chronic ethanol consumption on crypt cell production rate, birth rate, and proliferative compartment size in the distal colon of F344 rats

Treatment	Age (mth)	Crypt cell production rate (95% confidence limits) (cells/crypt/h)	Birth rate (cells/1000 cells/h)	Proliferative compartment (% of total crypt)
Control	2	4·3 (2·7 to 5·9)	9·5	41·5 (7·8)
	12	4·1 (2·7 to 5·5)	8·4	43·4 (5·6)
	22	4·5 (3·3 to 5·6)	8·1	42·9 (7·6)
Ethanol	2	5·8 (3·3 to 8·2)	13·3	36·8 (6·6)
	12	7·4 (6·0 to 8·7)*	15·3*	46·0 (6·9)
	22	10·7 (8·9 to 12·4)†‡	17·9†‡	44·6 (6·6)

*†Chronic ethanol consumption increases CCPR and birth rate significantly in 12 month (p<0·001) and 22 month old (p<0·001) rats compared with controls; ‡age increased CCPR significantly only in 22 month old compared with 2 month old ethanol fed rats (p<0·05).

TABLE II   *Effect of chronic ethanol consumption and age on crypt cell population in the distal colon of F344 rats*

Treatment	Age (mth)	Crypt column height (cells)	Cell column/crypt (n)	Total crypt population (cells)
Control	2	33·25 (2·5)	13·65 (2·7)	454
	12	33·87 (2·2)	14·24 (1·9)	482
	22	37·06 (2·9)*†	14·92 (1·6)	553
Ethanol	2	36·41 (2·8)	11·93 (1·3)	434
	12	36·33 (3·0)	13·25 (1·2)	481
	22	38·58 (2·3)	15·51 (1·0)‡§	598

*†Crypt column height was significantly increased in 22 month old control rats compared with 12 month old (p<0·05) animals; ‡§cell columns/crypt were significantly increased in 22 month old ethanol fed rats compared with 12 month old (p<0·05) animals. Chronic ethanol consumption had no significant effect on cell population.

and age is maintained. Although the birth rates of cells in the rectal crypt is strikingly enhanced by chronic ethanol consumption, this was not associated with a significant extension of the proliferative compartment.

Table II shows histologically determined crypt sizes that were not significantly influenced either by ethanol or by age. There seems to be a trend, however, towards larger crypts with increasing age. Age and chronic ethanol consumption did not exhibit a significant effect on CCPR in the proximal colon. Chronic ethanol feeding did not influence CCPR (95% confidence limits) in 2 month old (4·1 (2·8–5·4) v 4·6 (3·0–6·0) cells/crypt/ hour), in 12 month old (4·4 (3·0–6·0) v 3·9 (1·0–6·8) cells/crypt/hour), and in 22 month old (3·6 (1·4–5·4) v 2·9 (2·0–3·7) cells/crypt/ hour) rats.

Figure 2 shows a significant correlation between CCPR and the respective mucosal acetaldehyde concentrations after an acute ethanol dose in various colonic sites. The colonic site with the highest CCPR (distal colon of 22 month old ethanol fed rats) showed also the highest acetaldehyde concentration after an acute dose of ethanol, while the proximal colon of all three age groups exhibited the lowest CCPR and also the lowest acetaldehyde concentrations.

Discussion

The data presented clearly show that chronic

ethanol consumption stimulates rectal cell turnover, and that this stimulation is increased with age leading to a strikingly enhanced rectal cell regeneration especially in advanced age. A similar alcohol effect on rectal cell proliferation has been reported in earlier studies using Sprague Dawley rats with an age of 4–6 months.[12][13] In these experiments[13] and in those of others[20] the activity of ornithine decarboxylase, the trigger enzyme of cell replication, was also found to be significantly increased after chronic ethanol ingestion. As fermentable carbohydrates increase rather than decrease cell regeneration in the colon,[21] it is probable that the stimulating effect on cell regeneration results from ethanol and not from the carbohydrate deficient content of the alcohol diet.

The stimulation of mucosal cell turnover by alcohol, however, was found only in the distal and not in the proximal colon confirming earlier results from our laboratory.[12][13]

The vincristine induced metaphase arrest technique used in this study is considered to provide an accurate and direct measure of rates of intestinal cell production.[22][23] Kinetic studies using ^{3}H-thymidine are affected by various parameters such as the duration of the S phrase of the cell cycles and the availability of thymidine, which depends on thymidine pool size, the activity of thymidine metabolising enzymes, and the thymidine transport through the membranes.[23] All these processes can be influenced by either age or chronic alcohol consumption. Therefore the metaphase arrest technique seems to be ideal for studies of intestinal cell production as a function of nutritional manipulations and of age. This study has been performed in F344 rats up to 22 months of age. At this age Fischer 344 rats are considered senescent as they are within 6–8 months of their maximum life span.[24]

Using three different age groups, we provide evidence that alcohol increases rectal cell regeneration with age. In contrast with Holt and Yeh,[14] we did not find an age effect in itself on CCPR in the colon. In their experiments the important age effect was detected when stimuli such as starvation or refeeding were performed. There was also an increase in CCPR in the distal colon of 26–28 month old rats compared with 3–4 month old animals.[14] This increased CCPR mainly resulted from an increased crypt cell number with age and to a far lesser degree to an increased metaphase accumulation rate. In this context it has to be pointed out that the CCPR between the two studies cannot be directly compared as Holt and Yeh[14] estimated it from histological two dimensional data whereas we used directly obtained data from microdissected whole crypts. Our findings of lower absolute values of CCPR compared with Holt and Yeh[14] possibly result from liquid diet administration. It is well known that the consumption of liquid low bulk diets results in lower rates of mucosal cell proliferation.[25][27] Thus, the colorectal mucosa is probably in a hypoproliferative state.

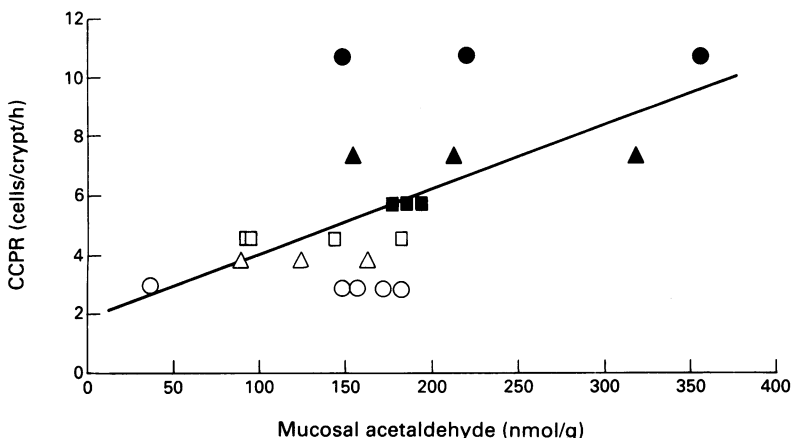While in earlier studies using Sprague

Figure 2: Correlation between crypt cell production rate (CCPR) and mucosal acetaldehyde concentrations in the proximal (open symbols) and distal (closed symbols) colon of young (2 month old, ■□), middle aged (12 month old, ▲△), and old (22 month old, ●○) male F344 rats measured 165 minutes after the acute intraperitoneal administration of ethanol (2·5 g/kg body weight).

Dawley rats we have detected higher colonic CCPR in the proximal compared with the distal colon of control animals[12] in this study using F344 rats the CCPR was in general lower and there was no difference in cell regeneration between the two intestinal segments. This may be because of the different strains of rats used in the different experiments. It is of interest that changes of rectal cell proliferation with age were most obvious when the mucosa was challenged by dietary stimuli such as alcohol as reported here or by starvation and refeeding as reported by Holt and Yeh.[14] Thus, aging may change the response of the mucosa to dietary manipulations and the mucosa may become more vulnerable with age. A similar increase in cell regeneration after chronic ethanol consumption has been recently reported in the rat oesophagus with the same diet and the same technique as described here.[28] As in earlier studies[12] the proliferative compartment of the crypt was found to be extended in 12 and 22 month old rats after alcohol ingestion. This did not, however, reach the value of statistical significance.

It has been suggested that acetaldehyde, the first and most toxic metabolite of ethanol,[29] may participate in the pathogenesis of increased rectal cell regeneration after ethanol consumption. It has been shown that acetaldehyde can be produced from ethanol in the colorectal mucosa by alcohol dehydrogenase[30 31] and by a microsomal ethanol oxidising system.[32] In addition, faecal bacteria can metabolise alcohol.[33] Acetaldehyde concentrations can be measured in the colorectal mucosa after an acute application of ethanol.[13 34] Higher concentrations of acetaldehyde are detected in the distal compared with the proximal colon and this difference is thought to result from the increased number of bacteria present in the rectum compared with the caecum[13] that can metabolise ethanol. In addition, mucosal alcoholdehydrogenase activity was also found to be higher in the rectum than in other colonic sites.[35]

It was also suggested that acetaldehyde possibly generated from ethanol by faecal bacteria may injure the colonic mucosa. Morphological changes seen in the rectal biopsy specimens from alcoholic patients[36] as well as a reduced life span of functional epithelial cells after chronic ethanol ingestion[12] may be indicative of such a toxic event. The data presented here further support the participation of acetaldehyde in colorectal cell regeneration, as mucosal acetaldehyde concentrations correlated significantly with CCPR in the colorectum. Thus, chronically generated acetaldehyde after longterm ethanol ingestion may lead to the mucosal tissue injury especially in the distal colon or rectum where more acetaldehyde is produced compared with more proximal large intestinal segments. Such injury is possibly associated with a secondary compensatory hyperproliferation as a response to tissue damage. As aging not only resulted in an enhanced effect of chronic ethanol feeding on CCPR, but also in higher acetaldehyde

concentrations after an acute dose of ethanol in the distal colon, it seems likely that acetaldehyde participates in this process. It is not clear from this study, however, why acetaldehyde concentrations were found to be higher in the rectal mucosa with age. Whether the bacterial flora changes with age possibly leading to a change in bacterial acetaldehyde metabolism needs to be determined. Another possibility to explain this finding could be a decrease of acetaldehyde metabolising enzymes with age as recently reported for the liver.[37]

Our data are relevant not only to the aging process but also potentially to the development of rectal cancer, which increases in frequency with advancing age[11] and after chronic ethanol consumption.[7-10] As already pointed out conditions that lead to enhanced colorectal cell regeneration also stimulate chemically induced carcinogenesis in the large intestine.[3 4] Experimental studies using the primary locally applied carcinogen acetoxymethylmethylnitrosamine give direct evidence that chronic ethanol ingestion enhances rectal carcinogenesis by an acetaldehyde mediated mucosal hyperproliferation.[13] Thus, rectal hyperregeneration after chronic alcohol ingestion could by itself favour carcinogenesis, which is especially relevant in old age.

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