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review

The dimethylhydrazine induced colorectal tumours in rat - experimental colorectal carcinogenesis

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Animal models of colorectal carcinogenesis represent invaluable research tool for investigating colorectal cancer (CRC). Experimentally induced tumours in laboratory animals provide opportunity for studying certain aspects of tumours that cannot be effectively studied in humans. Significant information on human CRC aetiology or factors influencing it has derived from studies using dimethylhydrazine (DMH) model that is one of the experimental models appreciated for its morphological similarity to human CRC. Today, DMH model represents useful research tool for the studies of colon carcinogens and chemopreventive agents. The review offers insight into morphogenesis and genetic alterations of DMH induced colorectal epithelial tumours in rats.

Key words: colorectal neoplasms - chemically induced; azoxymethane; 1,2-dimethylhydrazine; disease models, animal; rats

Introduction

The beginnings of the first animal model appreciated for its macroscopic and histological similarity to human colorectal carcinoma (CRC) extend to 1963, when Laqueur discovered that rats fed cycasin, a plant product, developed intestinal cancer. The active substance was identified and soon a similar compound, methylazoxymethanol acetate (MAMA) was synthesized that was more effective than the natural product. In 1970 Druckrey found that two chemicals structurally related to MAMA, dimethylhydrazine (DMH) and azoxymethane (AOM), were even more potent intestinal carcinogens.[1]

Today, DMH and its metabolite AOM are the agents widely used in experimental models of colorectal carcinogenesis in rodents. They are highly specific indirect colorectal carcinogens that induce the initiation and promotion steps of colorectal carcinogenesis yielding colorectal tumour lesions in a dose-dependent manner in rats, mice and hamsters.[2-4] In rats they can produce colorectal tumour lesions in almost 100% of treated animals.[4-8] Nevertheless, various strains of rats differ in susceptibility to these carcinogens.[8-10]

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Decreased susceptibility was reported also in female rats.[11,12] In chemically induced colorectal studies mostly 6-10 weeks old male rats are used and most often-applied rat strains are Fisher, Sprague-Dawely and Wistar (Table 1).

DMH metabolism

DMH is highly specific colorectal carcinogen that is metabolically activated in liver by series of reactions through intermediates azomethane, AOM and methylazoxymethanol (MAM) to the ultimate carcinogenic metabolite, highly reactive methyldiazonium ion.[13] MAM is excreted into the bile and transported to the colon or enter directly into epithelial cells of the colon from the blood circulation.[2,13,14] Some studies have demonstrated that rat colon epithelial cells are capable of metabolising DMH into carcinogenic metabolite without previous metabolism by other tis-

Table 1. Protocols used for chemical induction of colorectal lesions

References	C	Strain, sex and initial age or weight	Dose	R	N	D
Rubio *et al.* 1986	DMH	Sprague-Dawley *(male, 200g)*	21 mg/kg	s.c.	1	32
McGarrity *et al.* 1988	DMH	Sprague-Dawley *(male, 220-260g)*	20 mg/kg	s.c.	20	30
Park *et al.* 1997	DMH	Wistar *(male, 8-10 weeks)*	15 mg/kg	s.c.	19	24
Onoue *et al.* 1997	DMH	Fischer *(male, 10 weeks)*	20mg/kg	i.p.	2	11 34
Ghirardi *et al.* 1999	AOM	Fischer *(male, 6 weeks)*	15 mg/kg	s.c.	2	6 36
Rubio *et al.* 1999	DMH	Sprague-Dawley *(male, female, 200g)*	21 mg/kg	s.c.	27	32
De Jong *et al.* 2000	DMH	Sprague-Dawley *(male, 6 weeks)*	30 mg/kg	p.o.	5	24
Bissonnette *et al.* 2000	AOM	Fischer *(male, 80-100g)*	15 mg/kg	i.p.	2	37
Narahara *et al.* 2000	AOM	Wistar *(male, 6 weeks)*	7,4 mg/kg	s.c.	5	45
Ravnik-Glavac *et al.* 2000	DMH	Wistar *(male, 9 weeks)*	20 mg/kg	s.c.	15	25
Yamada *et al.* 2000	AOM	Fischer *(male, 6 weeks)*	15 mg/kg	s.c.	3	10
Takahashi *et al.* 2000	AOM	Fischer *(male, 6 weeks)*	15 mg/kg	s.c.	2	36
Kishimoto *et al.* 2002	AOM	Fischer *(male, 6 weeks)*	15 mg/kg	s.c.	3	4 4
Rodrigues *et al.* 2002	DMH	Wistar *(male, 6 weeks)*	40 mg/kg	s.c.	2	30
Veceric *et al.* 2004	DMH	Wistar, Fischer *(male, 8-10 weeks)*	25 mg/kg	s.c.	20	25

Legend: C, carcinogen; R, route of application (s.c., subcutaneous; i.p., intra peritoneal; p.o., per oral); N, number of applications; D, duration of experiment (weeks)

sues or colon bacteria.[15,16] Although intestinal flora[17,18] and bile acids[19] have influence on the incidence of tumours, the latter were induced also in germ-free rats[17] and function-isolated segments of rat colon.[14]

The ultimate carcinogenic metabolite of DMH is responsible for methylation of DNA of various rat organs including epithelial cells in the proliferative compartment of the intestinal crypts.[20] Metabolically activated DMH modifies not only nucleic acids but also histones and other DNA-binding proteins in the target cells.[21]

Tumour lesions induced by DMH

DMH is highly specific for colonic epithelium and induces tumours mostly in large bowel.[14,20,22] Colon specific susceptibility for this carcinogen is a result of a delayed or incomplete repair of damaged DNA in the colon compared to other organs,[20] leading to accumulation of mutations, and in a small proportion of cells giving rise to CRC. Higher susceptibility to colon versus small intestine has been shown in experiment where segments of colon that were transposed to the middle part of small intestine developed tumours but segments of small intestine that were transposed to the colon did not.[22] Tumours are distributed in all parts of the colon, but in a majority are observed in the distal part of colon.[4,8,23,24] Gross tumours are initially detected in the distal colon at 16 weeks but in proximal colon after 22 weeks.[23] The tumour incidence can be modulated by the amount of carcinogen administered and the number of applications. With increasing doses of the carcinogen, the latency period decreases and the tumour incidence increases.[3,4] Usually carcinogen at a dosage of 15-25 mg/kg body weight per week is administered subcutaneously (Table 1). In our studies DMH at a dosage of 15-25 mg/kg-body weight was injected subcutaneously once a week, for 15-20 weeks consecutively.[8,11,25,26]

Besides the colorectal tumours, the small bowel tumours are also induced but in much lower incidence.[5,8,11] However, small bowel tumorigenesis is characteristic of high-dose regimens of DMH.[27] Small intestinal tumours are mostly well or poorly differentiated adenocarcinomas.[5,8,11] Well-differentiated adenocarcinomas only occasionally demonstrate invasion through the intestinal wall and into the adjacent tissues.[5] On the other hand the poorly differentiated type is more aggressive and mostly metastasises to the mesenteric lymph nodes and in advanced stages frequently develops carcinosis of peritoneum or conglomerate tumours in the area between the duodenum, stomach, hilus of the liver and affected small intestine.[5,6]

Extraintestinal tumours may also be induced by DMH. Some rats develop tumours of Zymbal's gland (auditory sebaceous glands), usually squamous cell carcinoma.[5,11,28]

Colorectal tumour lesions

Aberrant crypt foci

The first specific morphologically identifiable lesions for colonic carcinogenesis are aberrant crypt foci (ACF). They were first identified in the colon of carcinogen treated mice by the light microscopic examination of the mucosal surface of colons that had been stained with methylene blue.[29] ACF are stereoscopically distinguished from normal crypts by their darker staining and larger size, elliptical shape, thicker epithelial lining, and larger perycriptal zone.[30-34] They appear within two weeks after carcinogen injection as single crypts that expand by crypt branching or multiplication. Sequential histologic analysis of ACF revealed that with time the number of ACF with increasing crypt multiplicity increases and a higher number of ACF

exhibit dysplasia.[30,32] It was observed that ACF with increasing crypt multiplicity are more resistant to apoptotic cell death.[31]

Hyperplastic ACF (Figure 1A) are composed of mixture of goblet and absorptive cells with enlarged or sometimes crowded nuclei without stratification. The luminal opening of ACF is slightly elevated from the surrounding normal mucosa and the crypts are elongated and occasionally branching with partial mucin depletion. Mitotic figures are limited to the lower two-thirds of the crypts and are never observed on the surface of ACF.[33,34]

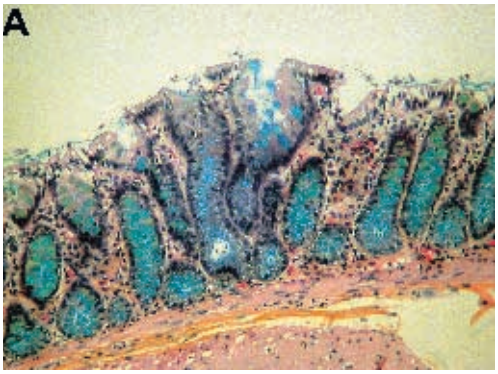
Dysplastic ACF (Figure 1B) are mostly composed of absorptive cells that display an unceasing proliferative activity.[33] Histologically these cells manifest cytoplasmic basophilia, a high nuclear-cytoplasmic ratio, prominent nucleoli and loss of cell polarity to variable degrees. The number of goblet cells is decreased and mucin depleted. The dysplastic crypt so formed tends to have an increased diameter, relatively smooth contour and dilated cryptal lumen in the lower half, and some irregularity and tortuosity with occasional evagination of the lining epithelium in the upper half.[33,34]

Adenomas and carcinomas

Two types of tumours can be distinguished grossly: polypoid (pedunculated or with a broad base) and non-polypoid (slightly elevated, flat or depressed).[5,35] Histologically, colorectal epithelial tumours are divided into adenomas and carcinomas.[36]

Adenomas are characterized by hypercellularity with enlarged, hyperchromatic nuclei, varying degrees of nuclear stratification, loss of polarity and decreased mucine excretion. Depending on the degree of glandular or villous complexity, extent of nuclear stratification and severity of abnormal nuclear morphology, dysplasia in adenomas can be divided into mild, moderate and severe (Figure 2A).

Tumours that penetrate through the muscularis mucosa into the submucosa are classified as carcinomas.[36] When no clear evidence



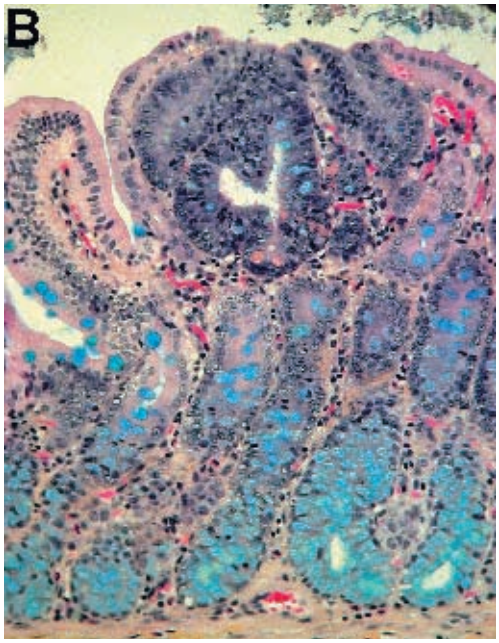


Figure 1. A. Hyperplastic aberrant crypt focus of colorectal mucosa. The focus is composed of three elongated crypts covered by slightly higher epithelium with nuclei at the base of cells. The luminal openings are elevated. **B.** Dysplastic aberrant crypt focus of colorectal mucosa. The focus is composed of epithelial cells with stratified, hyperchromatic nuclei, and with a loss of cell polarity and mucin secretion Ė the signs of dysplasia. Displacement of the surrounding normal crypts is evident.

of tumour growth through the muscularis mucosa is found additional criteria like sharp transition from unaltered epithelium to severe dysplasia, the presence of necrosis on the surface and desmoplastic stromal reaction are used.[37]

Carcinomas are divided into well, moderately and poorly differentiated adenocarcinomas (Figure 2B), mucinous adenocarcinomas (Figure 3A) if more than 50% of the lesion is composed of mucin and signet-ring cell carcinomas (Figure 3B) if more than 50% of tumour cells with prominent intracytoplasmic mucin are present.[36] Most frequently observed carcinomas in rat colorectal model are well-differentiated adenocarcinomas.[8,25,26] Some investigators[8,11,25] classify the stage of carcinomas according to Dukes staging system: stage A if tumour is limited to the intestinal wall, stage B if tumour grows through the lamina muscularis propria, stage C if tumour grows through the lamina muscularis propria and disseminates into the lymph nodes and stage D when carcinoma disseminates into distant organs.

Metastases

Metastases to the liver and lung are very uncommon in rats. The tumours that are capable of metastasis are almost exclusively the mucinous and signet ring cells carcinomas of the proximal colon. The adenocarcinomas of the distal colon have not been shown to metastasise. The metastases are generally

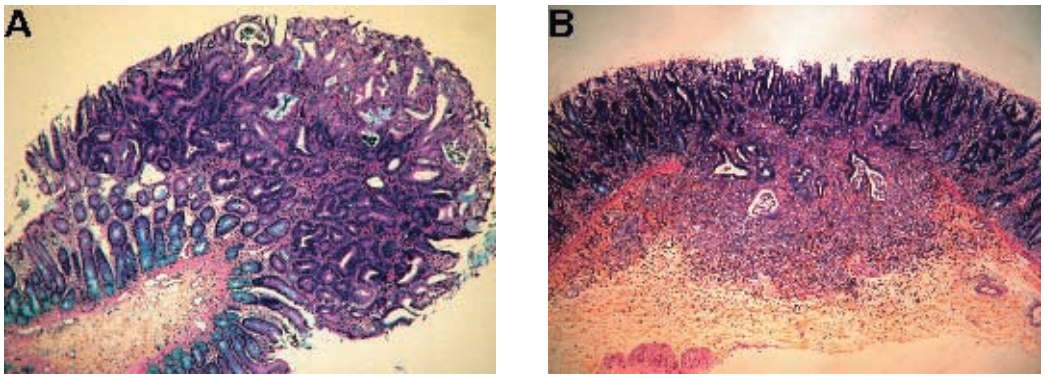


Figure 2. A. Polypoid tubular adenoma of colorectal mucosa with moderate grade of dysplasia. Muscularis mucosa is intact. **B.** A well-differentiated adenocarcinoma of colorectal mucosa. Submucosal invasion and accompanying fibroplastic stromal reaction is evident. Stage Dukes A.

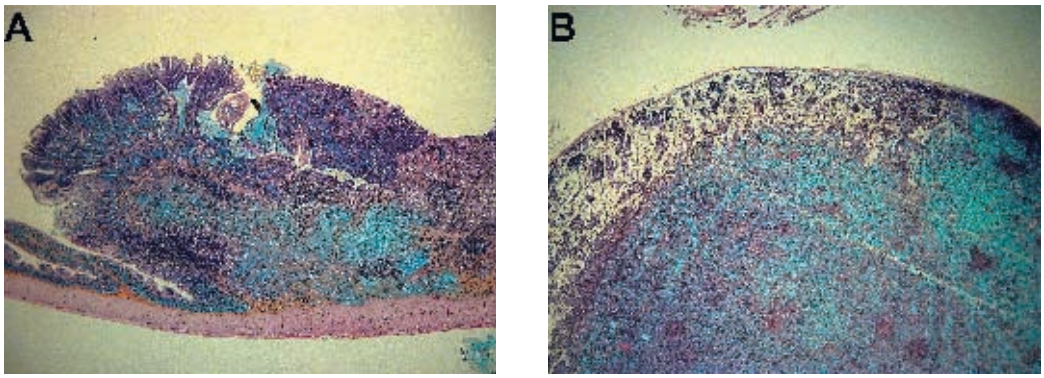


Figure 3. A. A mucinous carcinoma with a wide infiltration of submucosa. Note abundant extracellular mucin secretion. **B.** Signet-ring cell carcinoma metastasis in regional lymph node. Stage Dukes C.

found in regional lymph nodes (Figure 3B) or on the peritoneal surface.[5,11]

Tumour association with gut lymphoid tissue

Often, the earliest dysplastic mucosa is found over a mucosal lymphoid aggregate.[8,35,38,39] Significant association between tumour development, particularly non-polipoid adenomas[35] and mucinous adenocarcinomas,[38] and the presence of lymphoid aggregate have been observed. Hardman *et al*[39] have demonstrated that the association is due to higher proliferative activity in colonic crypts over the lymphoid aggregates.

Adenoma-carcinoma sequence and de novo formation of colorectal carcinoma

Several investigators[3,7,24,32,40,41] have observed differences in the histopathological findings of the carcinomas between the distal and the proximal colon in rats. The studies suggest that chemically induced carcinogenesis in the rat colon follows two distinct pathways: adenoma-carcinoma sequence, where histogenesis follows the ACF-adenoma-carcinoma sequences and *de novo* sequence where adenocarcinomas develop without passing through ACF stage.[3,7,24,40] The former is characteristic for middle and distal colon whereas the latter leads to the development of poorly differentiated, mucin-secreting carcinomas in the proximal colon.[3,7,24]

Molecular alterations

Mutations in the adenomatous polyposis coli gene (Apc), the gene coding for β-catenin (Ctnnb1) and K-ras gene were detected in colorectal tumours of rats administered DMH or AOM.[42] Alterations of specific oncogenes and tumour suppressor genes play role at different stages of carcinogenesis process. In rat carcinogenesis an extensive genomic instability was found, that is the necessary step for the generation of multiple mutations underlying the occurrence of cancer.[25,43]

Mutations in Apc gene were detected exclusively in the mutation cluster region of Apc[44] and were found only in 18% of tumours and not in ACF, suggesting that mutations of the Apc gene are associated with the transition from ACF to adenoma and adenocarcinoma and not from normal mucosa to ACF.[45] In rat tumorigenesis β-catenin mutations are more frequent event than Apc mutations,[44] suggesting that consequent alterations in the stability and localisation of the protein may play an important role in this colorectal carcinogenesis model.[46] Mutation causes activation of the β-catenin-Tcf pathway resulting in the accumulation of β-catenin in the cytosol and nucleus. Most of the mutations occur as single nucleotide substitution within functionally significant phosphorylation sites on exon 3. The most common mutation in the early lesions is G:C to A:T transitions that is recognised as the representative mutation in rat colorectal tumours.[46,47] β-catenin gene mutations were detected in tumours and dysplastic ACF, none in hyperplastic ACF. Also alteration in expression and cellular localization of β-catenin and inducible nitric oxide synthase were observed in all dysplastic ACF, adenomas and adenocarcinomas, but not in any hyperplastic ACF.[48]

K-ras mutations are important early event in the progression of chemically induced colorectal carcinogenesis in rodents,[49,50] frequently detected in tumours, dysplastic ACF[51] and even in hyperplastic ACF.[48] The majority of K-ras mutations are identified in codon 12 and 13.[49,50] Constitutive activation of K-ras by point mutation occurs with a frequency of 40-60%. K-ras point mutations occur mostly as G to A transitions.[49]

In carcinogen induced tumours elevated expression of c-myc,[52] c-jun[44] and c-fos[30] were detected and increased expression of cyclin D1 were observed, particularly by muta-

tions in either K-ras or β-catenin.[42]

In a subset of carcinogen induced rat colorectal tumours without detectable K-ras mutations constitutively activated wild-type p21ras have been observed, presumably due to increased expression of c-erbB1 receptor and decreased expression of GTPase activating protein. Mitogen-activated protein kinase (MAPK) activation and cyclooxygenase-2 expression were increased in tumours with mutated or activated wild-type p21ras. Colonic tumours with activated wild type p21ras, like those with mutated p21ras, have increased activation of extracellular signal regulated kinase-1 and extracellular signal regulated kinase-2, presumably via the activation of Raf-1 and MAPK kinase.[42]

Long-term and short-term assays

Repeated injections of DMH are needed to induce irreversible molecular and histological alterations in rat colons leading to development of ACF, adenomas and carcinomas. Based on duration of experiment assays can be divided into short-term and long-term.

Short-term assays require 4-11 weeks to complete (Table 1). In that time only ACF are induced, which are identified by light microscopic examination of large bowel.[29,30] They are precancerous lesions that are used as intermediate biomarkers to predict the ability of a test agent to affect tumour outcome.[29-34,53] Based on ability to retard or induce the appearance of ACF, compounds are classified as tumour inhibitors or tumour promoters.[29,30,53] However, it is important to take into consideration that ACF are a heterogeneous group of lesions[33,34,53] not equally distributed in colon. Ghirardi *et al*[41] observed the majority of ACF in the middle colon. Also Rodrigues *et al*[32] reported that majority of ACF were observed in the middle and distal colon and that induction of ACF by DMH in the short-term assay was correlated with development of well-differentiated adenocarcinomas. Park *et al*[24] demonstrated, that ACF are marker lesions for colorectal tumours, but only in distal colon where tumours follow the adenoma-carcinoma sequence. Therefore, compounds, which appear to be effective in the short-term, are usually examined in long-term experiments.

In contrast to short-term assays, the long-term usually take 20-40 weeks to complete (Table 1). In that time ACF, adenomas and adenocarcinomas are induced, which are further examined to assess the effect of testing substances on colorectal tumorigenesis.

Conclusions

Studies on DMH model allow monitoring the step-wise development of CRC by examining the dissected colons of randomly selected animals from a group, at different time intervals, as the disease progresses and under defined experimental conditions. They have already produced much important information on histology and biochemistry of tumour development as well as on factors that retard or enhance tumorigenesis. Even today, DMH model represents invaluable research tool for studying the molecular events of CRC and for developing and evaluating of a variety of novel cancer chemopreventive agents.

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