Hindawi Publishing Corporation
Journal of Biomedicine and Biotechnology
Volume 2011, Article ID 473964, 14 pages
doi:10.1155/2011/473964

Review Article

Morphological and Molecular Alterations in 1,2 Dimethylhydrazine and Azoxymethane Induced Colon Carcinogenesis in Rats

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Received 14 September 2010; Revised 30 October 2010; Accepted 29 November 2010

Academic Editor: Andrea Vecchione

The dimethyhydrazine (DMH) or azoxymethane (AOM) model is a well-established, well-appreciated, and widely used model of experimental colon carcinogenesis. It has many morphological as well as molecular similarities to human sporadic colorectal cancer (CC), which are summarized and discussed in this paper. In addition, the paper combines present knowledge of morphological and molecular features in the multistep development of CC recognized in the DMH/AOM rat model. This understanding is necessary in order to accurately identify and interpret alterations that occur in the colonic mucosa when evaluating natural or pharmacological compounds in DMH/AOM rat colon carcinogenesis. The DMH/AOM model provides a wide range of options for investigating various initiating and environmental factors, the role of specific dietary and genetic factors, and therapeutic options in CC. The limitations of this model and suggested areas in which more research is required are also discussed.

1. Introduction

Colorectal cancer (CC) is one of the leading causes of cancer-related morbidity and mortality in humans in western developed countries [1]. In recent years, increasing attention has been paid to environmental and food components, with the hope of identifying its preventive or carcinogenic effects [2, 3]. Much effort has been dedicated to a search for natural or pharmacological preventive agents, which would block or attenuate CC process [4, 5].

There are several experimental models of CC, providing a wide range of options for investigating various initiating and environmental factors, the role of specific dietary and genetic factors, and therapeutic options in CC. These models, which can be broadly divided into induced and transgenic animal models, vary in their suitability for investigating each of these factors. Homologous recombination of random chemically induced mutagenesis has been used to generate a series of APC (+/−) mice that exhibit mutation in codons 474, 716, 850, or 1638 of the APC tumour sup-

recombination approaches have also generated a mouse model with a mutation in the DNA mismatch repair genes. Mlh1 (+/−), Mlh1 (−/−), Msh6 (+/−), and Msh6 (−/−) mice exhibit accelerated small intestinal carcinogenesis. These animal models have been generated to study familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) syndromes and are described elsewhere [6]. Among chemically induced animal models, 1,2 dimethylhydrazine (DMH) and azoxymethane (AOM) animal models are most frequently used [4, 7–9]. Induction of colon cancer by other chemical carcinogens, such as nitrosamines or heterocyclic amines is less frequently used [10–12], because DMH and AOM (DMH/AOM) are less expensive, more potent and more convenient to use [7, 9]. The DMH/AOM model of colon carcinogenesis is a valid, well-appreciated, and widely used model of experimental colon carcinogenesis. It shares many similarities to human sporadic CC, including similarities in the response to some promotional and preventive agents [4]. Today, it is a widely used model for the evaluation of environmental, dietary, and

used to study morphologic and molecular mechanisms of the multistage development of colon cancer in order to elucidate new targets for chemoprevention [12, 13].

A number of excellent reviews on animal models of colon carcinogenesis [6, 12], including chemically induced carcinogenesis [7, 13, 14], have recently been published. Various aspects of the applicability of the DMH/AOM animal model are demonstrated in these papers. However, in order accurately to identify and interpret alterations that occur in the colonic mucosa when assessing natural or pharmacological compounds in an animal model, understanding both the morphologic and molecular development of the CC process in this model is required. This paper, therefore, summarizes the present knowledge of morphological and molecular features in multistep development of CC in the DMH/AOM rat model and its similarities to human sporadic CC. In addition, features and limitations of this model and suggested areas in which more research is required are also discussed.

2. Induction of DMH/AOM Colon Carcinogenesis

DMH and its metabolite AOM are widely used agents for the induction of colorectal carcinogenesis in rodents. DMH is metabolically activated in the liver by a series of reactions through intermediates AOM and methylazoxymethanol (MAM) to the ultimate carcinogenic metabolite, highly reactive methyldiazonium ion [15]. MAM can be excreted into the bile and transported to the colon (the development of small intestinal tumours distal to the entrance of the bile duct into the intestine is ascribed to this path) or enter directly into epithelial cells of the colon from blood circulation [15]. Some studies have also demonstrated that rat colon epithelial cells are capable of metabolizing DMH into the carcinogenic metabolite without previous metabolism by other tissues or colon bacteria [16, 17]. The ultimate carcinogenic metabolite of DMH is responsible for methylation of the DNA bases of various organs, including epithelial cells in the proliferative compartment of the crypts, which result in a great loss of colonic cells by apoptosis, an increase in proliferation, and an apparent increase in mutations of colonic epithelial cells [18].

However, DMH/AOM are highly specific carcinogens that induce colorectal tumours in a dose-dependent manner [19]. Various rat and mouse strains differ in susceptibility to these carcinogens [12, 20]. The susceptibility for DMH/AOM-induced colorectal carcinogenesis is also sex [21] and age dependent [22, 23]. Most commonly, 6 weeks old male F344, Wistar and Sprague-Dawely rats are used [7, 8]. Colon carcinogenesis is usually induced by two s/c applications of DMH (150 mg/kg) or AOM (15 mg/kg) given one week apart. Using these protocols, animals are scored for intermediate biomarkers of colon carcinogenesis, termed aberrant crypt foci (ACF), 8–12 weeks after the application (short-term study) or for the number of colonic tumours 40 weeks later (long-term study). Chemopreventive treatment the initiation phase, during the promotion or progression phase, or through both phases. These protocols are used to assess the promotional or protective effects of the tested factor and when followed closely provide data that are quite reproducible (protocol explained in detail by Femia and Caderni [7] and Bird [9]).

Nevertheless, tumour outcome depends on the total amount of carcinogen administered and the latency period. In long-term studies, therefore, DMH is frequently administered weekly for 15 weeks in a relatively low concentration (20 mg/kg). Repeated injections of carcinogen are evident at a later stage of cancer development and not in the stage of preneoplastic lesions, that is, ACF [24] and result in higher tumour incidence and multiplicity than following one or two injections of a colon carcinogen [19, 24].

3. Histopathological Nomenclature of Colorectal Epithelial Lesions

DMH/AOM colon carcinogenesis is a multistep process with morphological and histological features similar to those seen in human sporadic colon carcinogenesis [25, 26]. It is widely accepted today that the adenoma to carcinoma sequence is characterized by recognizable histological changes that start with dysplastic aberrant crypts or intraepithelial neoplasia (IEN) [27, 28]. These lesions then have the potential to progress to advanced adenomas, which have a significant potential to transform into adenocarcinomas [26].

3.1. Intraepithelial Lesions. The first lesions in the multistep development of CC cannot be seen grossly. They can be identified in histological sections of colon mucosa after careful histological examination as hyperplastic or dysplastic epithelial lesions [29] or on the surface of whole mount colon under low-magnification stereomicroscope as ACF [24, 25] or mucin depleted foci (MDF) [28, 30]. It is important to bear in mind that visualization and identification of ACF or MDF on whole mount colon does not yield specific information on the histological features of these lesions. The structural and cytological features of ACF or MDF can be recognized or confirmed only after histological examination. Nevertheless, in order better to understand the histological background of ACF and MDA (which are described in the next section) as well as molecular alterations recognized and described at different stages of colon carcinogenesis, we will briefly summarize the histological criteria and classification of colorectal epithelial lesions in rodents, which share many similarities with human pathology [26, 29].

3.1.1. Hyperplastic Intraepithelial Lesions. Hyperplastic epithelial lesions are composed of a mixture of goblet and absorptive cells, with enlarged or sometimes crowded nuclei without stratification. Mitotic figures are limited to the lower two thirds of the crypts and are never observed on the surface of crypts. Nuclei are basally located, ovoid or round, with occasional visible nucleoli and usually uniformly dark. The luminal opening of crypts is slightly elevated

elongated and occasionally branching, with partial mucin depletion [29]. It is worth mentioning that hyperplastic epithelium has never been observed in colonic tumours of the DMH/AOM rat model. It has only been found in intraepithelial lesions composed of a various number of crypts. The role of hyperplastic aberrant crypts in the process of colon carcinogenesis in DMH/AOM models is not clear and is a matter of debate and further investigation.

3.1.2. Intraepithelial Neoplasia/Dysplasia. Inraepithelial neoplasia is a histological term for dysplastic lesions in the epithelial layer of colon mucosa that cannot be identified macroscopically but only after careful histological examination. The presence of dysplasia is regarded as early histopathological changes in the precursor lesions of colon cancer. The word dysplasia is used to describe structural and cytological alterations in the epithelium that predispose an organ to cancer development. IEN is synonymous with the terms atypical hyperplasia, microadenoma, carcinoma *in situ,* and dysplasia. Depending on the cytological and architectural features, IEN is classified as low grade or high grade. The differential criteria involve hypercelularity with enlarged, hyperchromatic nuclei, varying degrees of nuclear stratification, loss of polarity, high nuclear/cytoplasmic ratio, nuclear crowding, increased mitotic index, and decreased mucine excretion [29].

3.2. Tumours. Pathological changes that can be seen macroscopically on the surface of colon mucosa as pedunculated or broad-based, slightly elevated, flat, or depressed (sessile or nonpolypoid) masses/lesions are called tumours. The incidence of colon tumours is the most reliable endpoint for evaluation of the chemopreventive effects of substances. Further histological examination is required to determine the malignant or benign character of tumours.

3.2.1. Adenomas. Tumours confined to the mucosa are benign neoplasms that are called adenomas. On the basis of the histologic type, adenomas can be tubular (when more than 75% of the adenoma is composed of branching tubules), villous (more than 75% of adenoma is composed of villous structures), or tubulovillous (25%–75% of adenoma composed of both tubular and villous structures). Depending on the degree of dysplasia on the most severely dysplastic area of each tumour, adenomas are graded as low or high [29].

3.2.2. Adenocarcinomas. Tumours that penetrate through the muscularis mucosa are malignant lesions, histologically denoted adenocarcinomas (well, moderately, or poorly differentiated). Based on the histological type, they are further classified into tubular, tubulovillous, or villous adenocarcinomas (as with adenomas), mucinous adenocarcinomas (if more than 50% of the lesion is composed of extracellular mucin and signet-ring cell can be present), signet-ring cell adenocarcinomas (if more than 50% of tumour cells

undifferentiated carcinoma (if no glandular structure is present) [29].

4. Biomarkers of Colon Carcinogenesis

In the past, assessment of chemopreventive substances was based on the incidence of tumours. Since the development of tumours is a relatively lengthy process, taking around 6–8 months to develop in the DMH/AOM rat model, preneoplastic lesions can be used as biomarkers for assessing the risk of developing colon cancer or for identifying modulators of colon carcinogenesis in short-term studies [9]. The use of preneoplastic lesions as biomarkers was not possible until 1987, when Bird [31] developed a simple, rapid and cheap methodological approach to detecting ACF [25, 31]. In the last decade, additional biomarkers of colon carcinogenesis have been identified, such as dark ACF [32], flat ACF [33], dysplastic ACF [34], MDF [30], and β-catenin accumulated crypts (BCAC) [35]. Their characteristics and application in short-term studies are briefly described.

4.1. ACF. ACF are the first lesions in the development of CC that can be identified microscopically on the surface of the whole mount colon mucosa after methylene blue staining. They have been identified in carcinogen treated rodents [25, 31] and in humans at high risk of developing CC (personal or familial history) [36, 37]. A number of studies in rodents and humans, including molecular analysis, have shown that ACF are lesions that are a valuable intermediate biomarker in the development of colon carcinogenesis [38]. ACF have to date been used as an endpoint in identifying and assessing the preventive or promotional role of natural and pharmacological compounds, as well as dietary and environmental factors, in the process of colon carcinogenesis [4, 5].

An increasing number of studies have demonstrated that ACF in both animals and humans are a heterogeneous group of lesions containing multiple genetic, epigenetic, and phenotypic alterations [37–40]. Histologically, ACF exhibit variable features, ranging from mild atypia to severe dysplasia. Most ACF show a hyperplastic character, while only a small subgroup of ACF has been found to contain intraepithelial neoplasia (such as severe dysplasia, microadenoma, or carcinoma *in situ*). It has been shown that ACF with hyperplasia possess different genetic and epigenetic alterations than ACF with dysplasia, and some studies have suggested that ACF possessing hyperplastic feature may not be directly related to tumorigenesis [27, 28]. However, there are reports suggesting that some ACF possessing hyperplasic features may progress to ACF with dysplasia [40, 41].

Nevertheless, ACF are useful biomarkers for the screening of compounds for their chemopreventive activities [5, 42]. When using ACF as biomarkers, it is important to take into consideration that ACF are a heterogeneous group of lesions. The total number of ACF may be considered to be a valid biomarker only at a very early stage of carcinogenesis, while, in subsequent weeks, ACF with higher

a more specific biomarker than the total number of ACF. In more advanced stages of colon carcinogenesis, ACF may not be a reliable intermediate biomarker of colon carcinogenesis (explained in detail by Bird and Good [24] and Raju [42]). It is also important to mention that ACF are not equally distributed among the proximal, middle, or distal colon. The majority of ACF develop in the middle and distal colon [43–45], which needs to be taken into account when using ACF as biomarkers (comprehensively discussed by Bird [24, 25] and Raju [42].

4.2. Subgroup of ACF with Dysplastic Features (Dark, Flat, and Dysplastic ACF). Since dysplasia is widely accepted as an indication of an increased risk of progression to cancer, it has been suggested that dysplastic crypts may be more directly associated with tumorigenesis than ACF [27]. Dysplastic ACF have recently been identified by various investigators using different approaches. Ochiai et al. [34] developed a differential staining method to identify dysplastic ACF, while identification of flat ACF [33] and dark ACF [32] were based on the surface morphology of ACF. However, all these lesions have been observed as subgroups of ACF with thicker epithelial lining, compressed luminal openings and mildly enlarged crypts, which were not elevated from surrounding epithelium. Histologically, all these subgroups of ACF have possessed dysplastic features with absent or scarce mucin production and have shown cytoplasmic and nuclear accumulation of β-catenin [32–34]. Based on a description of surface morphology (except the description of staining intensity) and histological characteristics, it is likely that flat, dark, and dysplastic ACF may represent the same group of ACF with dysplastic features. If each of these lesions represents a different subgroup of dysplastic ACF, their use as biomarkers would probably be questionable or confusing. Further investigations and determination of their relations are certainly needed before they can be used as biomarkers.

4.3. MDF. MDF are identified on the mucosal surface of unsectioned colon after staining with high-iron diamine alcian blue (HID-AB), which visualizes crypts with mucous production [30]. Identification of MDF is based on a scarce or absent production of mucous, which is a common feature of severe dysplasia. In contrast to ACF, which are histologically heterogenous, MDF are composed of dysplasic crypts, which display frequent genetic and epigenetic alterations observed also in colon cancer [46–49]. It has been shown that MDF appear 7 weeks after carcinogen administration and their number and multiplicity increases with time. MDF have been demonstrated as a potential biomarker for evaluation of the chemopreventive effects of natural or pharmacological compounds in colon carcinogenesis [50]. Since few studies have evaluated MDF as a biomarker, further investigations are needed to evaluate their role in colon carcinogenesis.

4.4. BCAC. In contrast to ACF and MDF, which can be identified

Table 1: Nomenclature of colorectal lesions according to their morphological appearance on whole mount colon (low magnification) or according to their histological characteristics identified in embedded and stained colon sections under a high-magnification microscope.

Morphological description	Histological description
Tumour	Adenomas:
(i) polypoid	(i) low-grade dysplasia
	(ii) high-grade dysplasia
	Adenocarcinomas:
(ii) nonpolypoid	(i) well, moderate, and poorly differentiated adenocarcinomas
	(ii) mucinous adenocarcinomas
	(iii) signet-ring cell adenocarcinomas
	(iv) solid or undifferentiated carcinomas
ACF (methylene blue)	Intraepithelial lesions:
(i) "dysplastic"	(i) hyperplastic
(ii) dark	(ii) dysplastic/intraepithelial neoplasia/microadenoma/carcinoma in situ
(iii) flat	
MDF (HID-AB)	(a) BCAC (immunohistochemical staining)

ACF, aberrant crypt foci; BCAC, β-catenin accumulated crypts; MDF, mucin depleted foci; HID-AB, high-iron diamine alcian blue.

of BCAC is based on an immunohistochemical method in sectioned colon [35]. BCAC are intraepithelial lesions that accumulate β-catenin protein in the cytoplasm and nucleus and harbor frequent β-catenin (*Ctnnb1*) mutations. Histologically, BCAC shows dysplasia with reduced or absent mucin production [35, 51].

Based on the assumption that mutations in the β-catenin gene or accumulation of β-catenin are the necessary first step in rat colon carcinogenesis, crypts with increased β-catenin expression have been proposed as a more relevant biomarker of colon cancer than ACF [27, 28, 35, 51]. However, histological identification and quantification of BCAC is relatively costly, tedious, and time consuming, which limits the use of BCAC as a biomarker.

However, we do not know at present whether BCAC, MDF, dark, flat, and dysplastic ACF are related lesions. The development of these dysplastic lesions is clearly related to the early development of tumours. Histologically, all these lesions show dysplasia with scarce or absent production of mucin and accumulate β-catenin in the cytoplasm and/or nucleus. Accordingly, it is possible that all these lesions are only dysplastic subgroups of ACF, which may predict tumour outcome better than ACF itself. However, first investigations suggest that this assumption may not be correct, because these lesions may not overlap completely [52, 53]. Further investigations are, therefore, needed to elucidate their features, evaluate relations and discrepancy among them and to identify the reason for

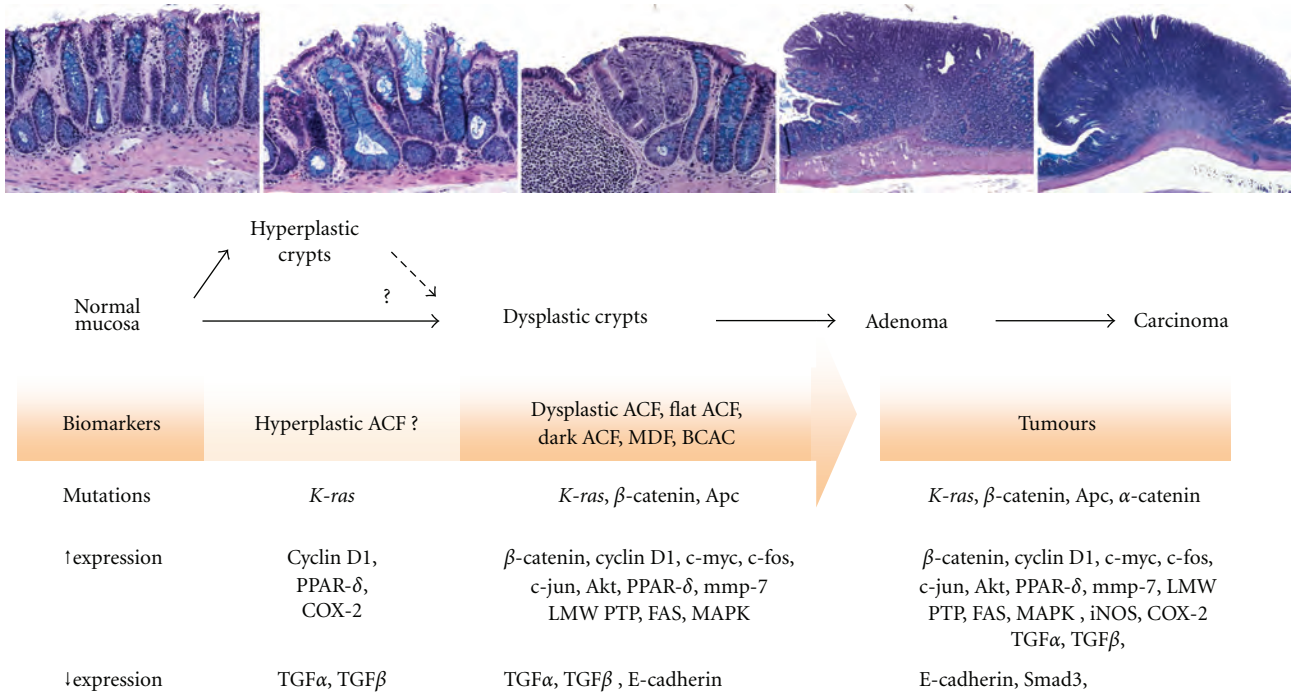

Figure 1: Phenotypic, genetic, and epigenetic alterations involved in multistep development of colon carcinogenesis in the DMH/AOM rat model.

5. Gene Mutations in DMH/AOM Colon Carcinogenesis

Colon carcinogenesis is a multistage process, involving multiple genetic and epigenetic changes that provide tumour cells with a selective advantage to expand their clones [54]. The stepwise development of CRC from dysplastic crypts, adenomas to carcinomas provides opportunities for the investigation and identification of molecular alterations at various stages of tumour development [24, 25, 55]. Genes that are mutated at different stages of colorectal carcinogenesis in human sporadic CC have been found to be also mutated in DMH/AOM-induced colon carcinogenesis and are described and discussed below.

5.1. Apc/β-Catenin. Mutations in the tumour suppressor gene, *APC*, are responsible for an inherited predisposition to colon cancer, FAP. *APC* mutations are also believed to be the earliest events in the formation of sporadic colon adenomas [54]. They have been identified in up to 80% of sporadic colon tumours in humans [56]. The most common *APC* mutation in human colon adenomas is *APC* loss of heterozygosity (LOH), which causes truncation of the protein and its inactivation. It is believed that the main function of APC is the regulation of free β-catenin in concert with glycogen synthase kinase 3β (GSK-3β) and other proteins [54, 57]. It has been found that half of human colon tumours with intact APC protein have a mutation in the β-catenin gene [26].

Apc mutations have also been identified in colorectal lesser degree and in a different region from that observed in humans [58]. In DMH/AOM-treated rats, up to 33% of colon tumours harbour *Apc* mutations. These *Apc* mutations have frequently been found located upstream from the region corresponding to the human *APC* mutation cluster region (nt 3,186–3,810; nt 3078 and 3835 in exon 15). They have mostly been missense or truncated point mutations (G to A and C to T transition) [58–60]. A deletion of the region containing the *Apc* gene has recently been found in one out of ten tumours, suggesting that LOH may also be involved in inactivation of *Apc* in this model [61].

In the DMH/AOM rat model, β-catenin mutations are a more frequent event than *Apc* mutations, occurring in up to 77% of tumours (Table 2). They are mainly point mutations (G to A transitions) localized in the GSK-3β phosphorylation consensus motif, which result in the inhibition of GSK3 β-dependent phosphorylation of β-catenin. However, *Apc* mutations or mutations in β-catenin have only been observed in the DMH/AOM rat model and in human CC in neoplastic/dysplastic lesions, that is, microscopic dysplastic epithelial lesions, adenomas, and adenocarcinomas (shown in Table 2) but not in hyperplastic lesions [35, 48, 58, 59, 62, 63].

5.2. K-Ras. The *K-RAS* gene encodes membrane bound protein with intrinsic GTPase activity, which is involved in the regulation of a number of important normal cellular functions, including proliferation, differentiation, and apoptosis. Single point mutations at specific sites within ras genes activate their oncogenic potential [64]. *K-RAS* mutations

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