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Murine Model for Colitis-Associated Cancer of the Colon

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Abstract

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), significantly increases the risk for development of colorectal cancer. Specifically, dysplasia and cancer associated with IBD (colitis-associated cancer or CAC) develop as a result of repeated cycles of injury and healing in the intestinal epithelium. Animal models are utilized to examine the mechanisms of CAC, the role of epithelial and immune cells in this process, as well as the development of novel therapeutic targets. These models typically begin with the administration of a carcinogenic compound, and inflammation is caused by repeated cycles of colitis-inducing agents. This review describes a common CAC model that utilizes the pro-carcinogenic compound azoxymethane (AOM) followed by dextran sulfate sodium (DSS) which induces the inflammatory insult.

Keywords

Inflammatory bowel disease; Colitis-associated cancer; Azoxymethane; Dextran sulfate sodium; Murine model; AOM/DSS model

1 Introduction

In many human pathologies inflammation and cancer development are known to be intertwined [1, 2]. Ulcerative colitis and Crohn's disease are two types of chronic IBD that increase the risk of development of CAC of the colon [3]. Both disorders are characterized by hyper-activation of the immune response, though they differ in the sites and specifics of the pathological features. The risk of developing CAC depends on multiple factors including the severity and longevity of the underlying inflammation as well as various known and unknown genetic predispositions. Moreover, the exact molecular mechanisms that underlie the transition from inflammation to cancer are not fully understood. The current body of evidence suggests that multiple factors play roles in CAC development: immune response, activation of oncogenes, inhibition of tumor suppressors, modifications to normal microRNA expression patterns, alterations to the epigenetic landscape, as well as commensal microbiota, and the corresponding inflammatory response of the epithelial cells [4].

Several animal models of CAC have been developed in rodents (mice, rats, and hamsters). The best studied chemically inducible model requires a combination of single injection of a

carcinogen [azoxymethane (AOM), a pro-carcinogen that is metabolized to methylazoxymethanol (MAM), the active agent, in the liver] that is followed by an inflammatory insult using dextran sodium sulfate (DSS) [5]. The AOM/DSS model produces a pathology manifested by severe colitis with loss of body weight and bloody diarrhea that is followed by development of multiple colon tumors. The exact location of the tumors along the length of the colon varies depending on the mouse strain and background [4]. The carcinogenic process of this system has a pathological progression from normal intestinal crypts to the formation of foci harboring aberrant crypts with crypt fission and finally to the emergence of microadenomas [4, 6, 7]. These steps recapitulate the sequence of CAC formation in humans from inflammation through dysplasia to carcinoma.

Furthermore, the molecular perturbations governing CAC development and progression in the AOM/DSS model also mimic those observed in human patients. The molecular hallmarks of CAC development following AOM/DSS treatment are described in detail in Table 1. Studies from this animal model have shown increased activity of the Wnt signaling pathway due to mutational activation of β-catenin resulting in its accumulation in the nuclei [5, 7, 8]. The increased Wnt signaling activity along with an enhanced inflammatory immune response (e.g., interleukin 6 (IL-6)) in this model has been shown to result in elevated levels of c-myc, an activator of cell cycle progression and known oncogene [9]. It has been shown that chronic inflammation changes the pattern of microRNA expression resulting in the activation of the phosphatidylinositide 3-kinase (PI3K) signaling pathway, aiding in the transition from inflammation to cancer [10]. Many key inflammatory components, nuclear factor of Kappa light polypeptide gene enhancer in B-cells (NF-κB), Janus kinase (JAK), signal transducer and activator transcription 3 (STAT3), pro-inflammatory cytokines (TNF alpha and IL-6), cyclooxygenase 2 (Cox-2; PTGS2), and inducible nitric oxide synthase (iNOS), have been demonstrated to be increased and activated during CAC formation [3, 7, 8, 11 – 14]. Upon chronic inflammation the immune system displays elevated levels of lymphocyte, plasma cell, and macrophage infiltration into the site of injury, all of which have the potential to modify the inflammatory microenvironment of the intestine and promote tumor establishment [6]. Additionally, recent studies demonstrated that AOM/DSS treatment causes a significant alteration in the diversity of the microbiome, promoting its dysbiosis and nurturing cancer development by promulgating the inflammatory environment [15]. Related to this, it is worth noting that some studies use *ApcMin/+* mice [16].

As with any model, the AOM/DSS-induced CAC model has its limitations. For example, *Kras* or *p53* mutations are typical in human, but have not been detected in this mouse model [17]. In contrast, *Kras* mutations have been observed in rats using the colon cancer model of AOM alone [18, 19]. Another factor is diet that significantly affects the outcome of AOM/DSS-induced CAC. Some of the altered effects may be due to changes in microbiota, an area of rapidly expanding research. The role of microbiota in this process is not well understood; however, alterations in host microbiota may account for inter-institutional variability. Helicobacter is also an additional factor that should be considered, as this can manifest and exacerbate the colitis symptoms of this model.

Rodent models for CAC serve as powerful tools for the investigation into mechanisms by which tumors develop in an inflammatory setting. The samples collected from these models can be analyzed for pathology [20, 21], immune infiltration, and immunohistochemistry for signaling pathways, specific immune cell populations, and genes of interest. Molecular biology techniques can be used to examine gene expression by quantitative RT-PCR and RNA sequencing, as well as by microarray and DNA sequence analysis. Protein profiling can be complemented by western blotting and other advanced proteomic techniques. Chromosomal and microsatellite instability and epigenetic changes can also be examined from tissues collected using this model. AOM/DSS-induced CAC can be utilized in combination with potential novel therapeutic modalities in order to determine and validate novel therapeutic targets.

In summary, the AOM/DSS animal model closely recapitulates histological, pathological, and molecular features of CAC in humans. Thus, it is a suitable model for studying the development, progression, and chemoprevention of this disease.

2 Materials

1. Male or female C57BL/6 mice (C57BL/6J, The Jackson Laboratory or Charles River ex-USA), 8–12 weeks old (*see* **Note** [1]). All animal experiments have to be done in accordance with institutional and national guidelines and regulations.
2. Azoxymethane (AOM) (Sigma, cat. #: A5486-100MG).
3. Dextran sulfate sodium (DSS), 36–50 kDa (MP Biomedicals, cat #: 160110 (SKU 0216011050)) (*see* **Note** [3]).
4. Acetic acid 100 %.
5. Ethanol 100 %.
6. 10% Buffered formalin.
7. Modified Bouin's fixative (prepared in-house; *see* Subheading 3.2).
8. 1 mL Syringes.
9. 10 mL Syringe.
10. Round-tip gavage needle.
11. 28 × 3/4 Needles.
12. Dissection tools.
13. Dissecting microscope.
14. Wooden toothpicks.

[1]While mice from both sexes are susceptible to AOM/DSS-induced CAC, based on author's observations, female mice tend to yield more consistent and reproducible results. An exception to this is for studies involving dietary manipulations, including high-fat diets; these studies typically utilize male mice.

[3]The source of DSS is very important as DSS from different manufacturers or even different lots can yield significantly different results. It is recommended to utilize the same lot from the same manufacturer for each experiment. DSS should also be prepared fresh for each cycle of administration, as the solution may become turbid after several days of administration.

15. Eppendorf tubes.
16. Chamber connected to a gas source of CO_2 or isoflurane.
17. Ruler (in centimeters).
18. Reagent, such as Hemoccult SENSA kit (Beckman Coulter, cat. # 64151), to detect occult blood in stool.
19. Weighing scale sensitive enough to weigh mice (e.g., sensitivity range 0.01–100 g).
20. Clean 150 mm Petri dishes.
21. Phosphate-buffered saline 1× (PBS).

3 Methods

3.1 Preparation of AOM for Injection

Reconstitute AOM to a final concentration of 10 μg/μL by adding 10 mL sterile deionized water (dH_2O) to the AOM vial. Vortex until dissolved. Aliquot about 500 μL in Eppendorf tubes and store in −20 °C until use (*see* **Note** [2]).

3.2 Preparation of Modified Bouin's Fixative

To prepare 1 L use 1 L measuring cylinder and add 500 mL ethanol 100 %, plus 50 mL acetic acid 100 %, and then add 450 mL dH_2O. Final concentration of ethanol and acetic acid is 50 % and 5 %, respectively. Stir to mix. Store at room temperature. (This modified fixative contains no picric acid or formaldehyde.)

3.3 Preparation of AOM and Injection in C56BL/6 Mice

Weigh mice, and then per mouse, prepare 10 μg AOM/g mouse in a total volume of 100 μL to be injected (*see* **Notes** [2] and [4]). Example: for a 20 g mouse take 20 μL of the 10 μg/μL AOM stock plus 80 μL sterile dH 2 O, i.e., a total of 100 μL. To compensate for losses due to dead needle volume, per mouse take 60 μL of the AOM stock + 240 μL sterile dH_2O = 300 μL, and inject 100 μL i.p. using a 28G needle and 1 mL syringe.

3.4 Preparation and Administration of DSS

1. One week after AOM injection, prepare 2.5 % DSS by dissolving 2.5 g DSS per 100 mL dH_2O (*see* **Note** [3]). For each experiment prepare a total of 300–400 mL of 2.5 % DSS to be used per mouse cage. Weigh the mice on the first day of DSS administration, and every day after that until the experiment is terminated (*see* **Note** [4]).
2. Leave the mice with 2.5 % DSS in drinking water for 5–6 days, followed by 2 weeks of recovery, replacing the DSS water with regular water.

[2]Stock solutions of AOM should not be used after repeated freeze-thaw cycles, and should be replaced after 1 year.
[4]Body weight should be used as an indicator of overall animal health and DSS doses may need to be modified.

3. After the recovery period start a second cycle of 2.5 % DSS for 5 days followed by 2 weeks of recovery, replacing the DSS water with regular water.

4. Euthanize the mice at the end of the last of recovery, 2–6 weeks after the last round of DSS, which varies by background strain and genetic modification (*see* **Note** [5]).

3.5 Assessment of Colitis Induction in Treated Mice

1. By days 5–6 of the first DSS treatment, as an indicator of induction of colitis mice will have lost approximately 10 % of their starting body weight (*see* **Note** [4]).

2. If blood stains are not obvious around the anal region, then collect stool from mice to determine the presence of occult blood by using the Hemoccult kit. Working one mouse at a time, lift the mouse from its tail to elevate its hind legs. Hold the mouse in this position with one hand to induce the mouse to defecate. With the other hand hold a wooden spatula (provided with the kit) and collect on it any faecal material the mouse produces. Assess the consistency of the stool and determine the stool score (*see* Table 2). Follow instructions from the manufacturer to test for occult blood in stool.

3. By the end of the first recovery period, mice weight should return to approximately the level that it was before DSS treatment.

4. By days 5–6 of the second DSS treatment, mice should have lost about 10–15 % of their starting body weight as an indicator of colitis (*see* **Note** [4]).

5. Test for occult blood in stool as described above in **step 2**.

6. By the end of the second recovery period mice weight should return to approximately the level that it was before DSS treatment.

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[5]This chapter describes a model of CAC used primarily in C57BL/6 mice (C57BL/6 mice from any commercial vendor seem to exhibit similar sensitivity). This strain of mice is commonly used for this model as they are susceptible to AOM/DSS-induced CAC [22]. Suzuki et al. evaluated strain differences among murine models of AOM/DSS-induced CAC and determined that Balb/c mice were most susceptible to tumor development. In this study, Balb/c mice demonstrated the highest tumor incidence (100 %) followed by C57BL/6 (80 %), C3H/HeN (29 %), and DBA/2 N mice (20 %) [22]. Of note, this study was conducted using a single injection of AOM (10 mg/kg) as described here; however, mice received 1 % DSS in drinking water for 4 days (only one cycle) and were euthanized at 18 weeks. This study demonstrated that the background strain of mice used significantly affected tumor development. Listed below are some modifications that can be used with this model.

a. DSS doses can be increased (or decreased) based on the background strain of mice or genetic modification in mice for mice that are less (or more) susceptible to colitis. Mice that are very susceptible to colitis may need ≤1 % DSS.

b. The duration of the experimental model can be manipulated to increase or decrease tumor development. Cycles of 2.5 % DSS (or lower) and water may be repeated more than twice to decrease the duration of the model.

c. Additional carcinogens have also been used to induce the initiation of tumors including 1,2-dimethylhydrazine (DMH; AOM is a metabolite of DMH), and/or methylazoxymethane (MAM). DMH has long been used to study colorectal cancer and adenomas induced by this carcinogen often invade into the submucosa and muscularis, while tumors induced by MAM and AOM do not. When administered with rounds of DSS, DMH yields similar tumor incidence to AOM [23]. MAM is commonly used in combination with 1-hydroxyanthraquinone in rat models of CAC (reviewed in [24]).

3.6 Assessment of CAC Following AOM/DSS Treatment

1. At the end of the second recovery period (2–6 weeks following the last cycle of DSS), euthanize mice (one mouse at a time) using CO_2 or isoflurane asphyxiation. Put the mouse in the chamber connected to the gas source. Allow the gas to flow gas into the chamber until the mouse is unconscious and all movement has ceased. Take the mouse out and place it on a flat surface with abdominal side down and perform cervical dislocation to ensure and confirm death of the mouse. This is best done by using one hand to hold and place a pair of forceps on the neck of the mouse right behind the skull and then apply slight pressure against the neck. With the other hand hold the tail firmly and pull backwards while keeping pressure on the neck with the forceps. You should hear/feel the cervical spine dislocate.
2. Using dissection forceps and scissors, cut an incision in the skin of the abdominal side. With a pair of forceps, hold the skin at the incision and pull gently away from the abdominal muscle tissue. Use a pair of scissors to cut the abdominal skin and expose the abdominal muscles.
3. Hold and pull up the peritoneum with the forceps. Make sure not to be holding and pulling at the intestines. Carefully make an incision in the peritoneal tissue, and continue cutting away to expose the intestines.
4. Identify the colon and trace to the distal end where it joins the rectum/anus. With a pair of scissors, cut as close to the anal opening as you can.
5. With one hand or forceps hold the distal end of the colon, and using the other hand gently unravel the entire length of the colon from any mesenteric connective and/or fat tissue.
6. Identify the cecum (small pouch between the small and large intestine), and cut at where the cecum and the colon join (the proximal end of the colon) to free the colon.
7. Using a ruler, measure to the nearest mm and record the length of the colon.
8. Fill a 10 mL syringe with modified Bouin's fixative and attach a gavage needle to it. Insert the needle about half a centimeter in the anterior opening of the colon.
9. With the fingers of one hand hold the needle inside the colon by applying firm pressure on the colon; with the other hand holding the syringe apply gentle but consistent pressure to flush the contents of the colon using modified Bouin's fixative. This step allows simultaneous cleaning of the colon and immediate fixation. Use a petri dish to collect the flow-through waste. Fixation can be observed by the colon color turning opaque (*see* **Note** [6]).
10. Using scissors, cut open the colon lengthwise. Hold with a pair of forceps and rinse briefly in a petri dish containing PBS.

[6]Make sure not to apply too much pressure during flushing; otherwise the colon might burst open.

11. Use the top of a petri dish to place the cleaned and opened colon. Place the colon with the luminal side facing upwards. The luminal side can be easily identified by the variegations/ridges present at the proximal end of the colon. Place the lid with the colon under dissection microscope. Identify any tumor growth along the length of the colon and record the number of tumors (*see* **Note** [7]).

12. Keep the colon flat open and pull it with forceps from its proximal end towards the edge of the petri dish. Keep luminal side facing up and hold the proximal end with the forceps with one hand and with the other hand hold a toothpick. Wrap the edge of the proximal end around the toothpick using the forceps and slightly pinch the wrapped edge against the toothpick to hold it in place. Gently and slowly start rolling the toothpick with your fingers to roll the colon around the toothpick to form a swiss-roll. Once the entire colon length has been rolled up, use a pair of forceps to carefully slide the colon swiss-roll off the toothpick and into a tissue processing/embedding cassette. Place the cassette in 10 % buffered formalin for processing.

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[7]Under a dissecting microscope, examine the colon for the presence of tumors which should appear as abrupt outgrowth relative to the luminal surface of the colon, and with a relatively denser cellular composition. Size determination is done by measuring the longest and shortest surface diameters of the tumors and calculating the average surface diameter. This step is best done if the dissecting microscope is equipped with an ocular micrometer. Size (average diameter) will vary from ≤1 to ≥3 mm. The length of the experiment and the particular mouse genotype and background are variables that will determine the tumor number and size.

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Table 1

Effectors of the AOM/DSS animal model

Factor	Effects	Selected reference
Inflammatory cells (macrophages, lymphocytes, plasma cells)	Elevated number	
Nuclear factor κB	Increased activity Increased nuclear localization	[11, 12]
JAK/STAT3 pathway	Increased activity	[14]
Pro-inflammatory cytokines (TNFα and IL-6)	Increased levels	[13, 14]
β-Catenin	Mutations in codons: 32, 33, 34 Increased nuclear localization	[5, 7, 8]

Table 2

Assignment of clinical scores of colitis in C57BL/6 mice [23, 24]

Parameter/score	Weight loss (%)	Stool consistency	Occult/gross bleeding
0	None	Normal	Negative
1	1–5	–	–
2	5–10	Loose stool	Hemoccult positive
3	10–20	–	–
4	>20	Diarrhea	Gross perianal bleeding