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Mast cell modulates tumorigenesis caused by repeated bowel inflammation condition in azoxymethane/dextran sodium sulfate-induced colon cancer mouse model

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ABSTRACT

Mast cells infiltrate the inflammatory microenvironment and regulate the production of many pro-inflammatory cytokines and mediators of inflammatory cell production to promote tumor development and growth in intestinal lesions. Currently, there are insufficient studies of the mediators and signaling pathways regulated by mast cells that influence the pathogenesis of colon cancer in inflamed colon tissue. This study aimed to confirm the role of mast cells in the incidence and growth of colitis-associated colon cancer (CAC) and to identify inflammation-mediated factors and signaling pathways related to tumor development. CAC was induced by the administration of azoxymethane (AOM) and dextran sodium sulfate (DSS) in mast cell-deficient (WBB6F1/J-W/W^{V}) and mast cell–sufficient control (WBB6F1_+/+) mice. The results confirmed that mast cell-deficient mice exhibited less tumor formation than normal mice under the same conditions, and down-regulated expression of pro-inflammatory cytokines and mediators. Mast cells play an important role in tumor formation by regulating pro-inflammatory cytokines and inflammatory mediators in CAC, indicating that they can act as new targets for the prevention and treatment of CAC.

1. Introduction

Colon cancer has been diagnosed in an increasing number of patients worldwide, and its incidence is increasing every year because of westernized dietary habits and obesity [1]. Previous studies have shown that chronic recurrent inflammatory responses, such as chronic inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, are associated with the development and progression of colon cancer [2]. This suggests that one of the characteristics of the tumor microenvironment is inflammation and that chronic persistent inflammation is closely related to cancer development [3]. In the case of CAC, the cumulative incidence of colon cancer in patients with IBD was approximately 2%, 8%, and 18% in 10-, 20-, and 30-year disease periods, respectively, considering various factors, including the degree of inflammation and disease duration [4].

Mast cells are widely distributed around blood vessels, and when tissue is damaged or infected, chemical substances contained in granules are secreted, leading to a defense mechanism. Moreover, mast cells maintain homeostasis against damage and infection and have many innate and adaptive immune functions [5]. For a long time, many animal and human studies have shown that a high number of mast cells are observed around tumor cells or inflammation lesions [6]. These findings suggest an important role of mast cells in the progression and development of many tumors [7]. Mast cells are activated when high amounts of inflammatory mediators, such as cytokines, are released [8]. Moreover, they play an important role in IBD, and the number of mast cells increases in inflammatory bowel lesions in patients with IBD [9]. In addition, mast cells play a crucial role in the formation of intestinal polyps, and strong infiltration of mast cells has been reported in tumor tissues of CAC [10]. Therefore, we hypothesized that mast cells would

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serve as new targets for immunotherapy for the treatment of colorectal cancer.

Although mast cells are currently known as one of the regulators of inflammatory colorectal cancer, detailed experimental evidence for signaling pathways and mediators that mast cell deficiency suppresses colorectal cancer is still lacking so far. The purpose of this study is to investigate in detail the role of mast cells in the pathogenesis of colorectal cancer and the interaction of the immune system.

To investigate the role of mast cells in colorectal cancer, AOM and DSS, which are carcinogens of colorectal cancer that can induce colorectal cancer in mice lacking mast cells and colonic mucosa, were used [11]. Our study was the first to elucidate the signaling pathways acting by mast cells in CAC disease, thereby examining the potential for treatment of IBD and CAC due to the modulation of mast cells.

2. Material and methods

2.1. Materials and chemical reagents

AOM were purchased from Sigma-Aldrich (St. Louis, USA). DSS was provided by MP Biomedicals (Solon, USA). TRIzol reagent and cDNA synthesis kits were obtained from TaKaRa Bio Inc. (Tokyo, Japan). SYBR Green was purchased from Applied Biosystems (Foster City, CA, USA). VECTASTAIN Universal Quick Kit for immunohistochemical (IHC) analysis was purchased from Vector Laboratories (CA, USA).

2.2. Animals

Male mast cell-deficient mice (WBB6F1/J-W/W^{V}) (6-weeks-old, 21–22 g, n = 10) and mast cell–sufficient control (WBB6F1_+/+) mice (6-weeks-old, 20–22 g, n = 20) were provided by Central Laboratory Animal Inc (Seoul, Korea). All mice were fed a basal diet and tap water *ad libitum* and were maintained under controlled conditions (humidity, 50 ± 10%; temperature, 22 ± 1 °C; 12:12 dark/light cycle). Mice were acclimated to the animal facility for 1 week. All animal experiments were conducted with the approval of the Animal Experiments Committee of Wonkwang University (WKU14-71).

2.3. Experimental design (induction of CAC)

A schematic diagram for the induction of CAC is shown in Fig. 1A. For the experiment, mice were divided into three groups containing ten mice each: (1) WT$^{(-)}$ (normal control group, (+/+) mice); (2) WT$^{(+)}$ (AOM/DSS treated group, (+/+) mice); (3) W/W$^{V(+)}$ (AOM/DSS treated group, W/W^{V} mast cell-deficient mice). At the start of the experiment day (day 0), mice in the WT$^{(-)}$ group were intraperitoneally (i.p.) injected with normal saline, and mice in the WT$^{(+)}$ and W/W$^{V(+)}$ groups were injected i. p. with 12.5 mg/kg AOM. After 1 week, mice in the WT$^{(+)}$ and W/W$^{V(+)}$ groups were supplied with drinking water containing 2.5% DSS (36–50 kDa) for 5 days, followed by normal drinking water for 14 days. This cycle was repeated three times. Mice in the WT$^{(-)}$ group were administered the same volume of normal drinking water during the experiment. At the end of the experiment, all mice were euthanized using CO_2 under ketamine anesthesia.

2.4. General observations

The body weight of all mice was measured once a week during the experimental period. After sacrifice, complete colons (including proximal and distal ends and anus) were removed from all mice to measure the colon length. The segment from the cecum to the anus was removed and opened up along a horizontal line to assess tumor development (number and size) in detail.

2.5. Evaluation of the severity of CAC

To assess the severity of CAC, disease activity index (DAI) was monitored every week during the experimental period. We examined as described previously body weight, stool consistency, and the presence of gross blood in the stool or at the anus of all mice [12]. All of these factors were used to calculate the DAI. The DAI scores assessed as 0 (Stool consistency: Normal, Occult/gross bleeding: None), 1 (Weight loss (%): 1–5), 2 (Weight loss (%): 6–10, Stool consistency: Loose, Occult/gross bleeding: Occult blood), 3 (Weight loss (%): 11–15) and 4 (Weight loss (%): >15, Stool consistency: Diarrhea, Occult/gross bleeding: Gross blood).

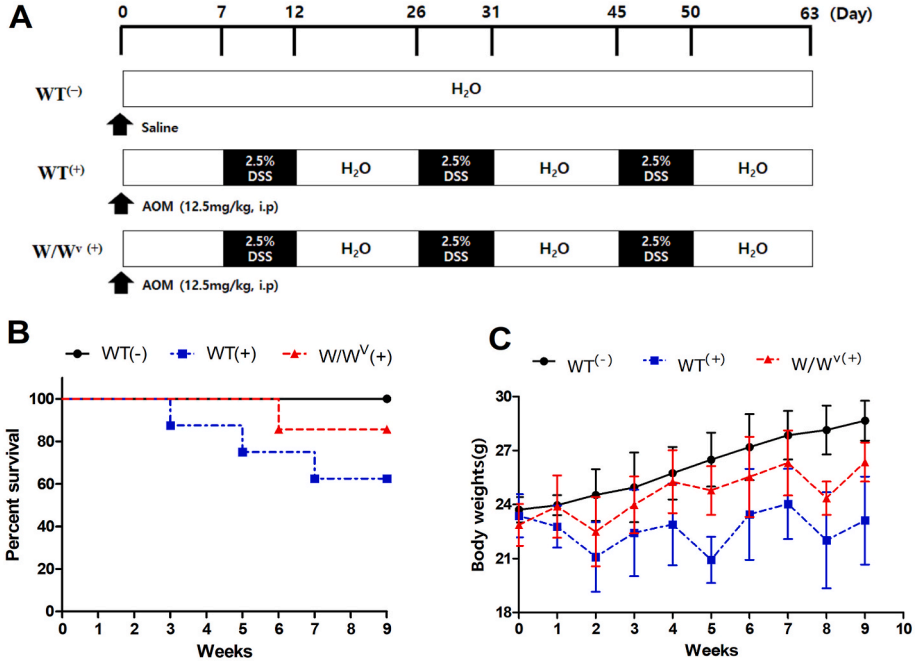

Fig. 1. Mast cell deficiency increases survival rate and body weight in AOM/DSS-induced CAC mice. (A) Schematic diagram of AOM/DSS-induced CAC mouse model. (B) Survival curves and (C) body weight curves of WT$^{(-)}$ (n = 10), WT$^{(+)}$ (n = 6), and W/W$^{V(+)}$ (n = 8) mice.

2.6. Hematoxylin and eosin analysis (H&E)

For histopathological examination, H&E staining was conducted using colon tissues of mice. Distal colon samples were immediately stored in 10% neutral buffered formalin. Colon tissues were dehydrated, embedded in paraffin, cut on a microtome in 4-μm thick sections, and stained with H&E. Images were captured using an EC3 digital color camera and CAS EZ imaging software. Neoplastic characteristics were scored based on the criteria listed as 0 (Number of lesions: 0, Crypts: Normal, Epithelium: Normal, Submucosal invasion: Absent), 1 (Number of lesions: 1–2, Crypts: Goblet cell depletion, Epithelium: Hyperplasia/ACF, Submucosal invasion: Present), 2 (Number of lesions: 3–5, Crypts: Branching, Epithelium: Low-grade dysplasia, Submucosal invasion: Present), 3 (Number of lesions: 6–8, Crypts: Complex budding, Epithelium: High-grade dysplasia, Submucosal invasion: Present), and 4 (Number of lesions: >9, Crypts: Branching, Epithelium: Low-grade dysplasia, Submucosal invasion: Present) [13].

2.7. Real-time PCR

The colon tissues of mice were homogenized with TRIzol reagent, and total RNA was extracted according to the protocol. After total RNA quantitation, cDNA was synthesized from extracted total RNA (2 μg) using the Super Script TM III kit. Real-time PCR was conducted using SYBR Green PCR Master Mix solution and a StepOne™ Real-Time PCR system to analyze the mRNA expression levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) [14] and inflammation-related genes (NF-κB, COX-2, and iNOS) [15]. GAPDH gene was used as an internal control, and primer sequences are listed as follow; mTNF-α: F-TAGCCAGGAGGGAGAACAGA, R-TTTTCTGGAGGGAGATATGG; mIL-1β: F-GCAACTGTTCCTGAACTCAACT, R-ATCTTTTGGGGTCCGTCAACT; mIL-6: F-GACAACCACGGCCTTCCCTA, R-GGTACTCCAGAAGACCAGAGGA; mNF-κB: F- GATGTGCATCGGCAAGTGG, R-AGAAGTTGAGTTTCGGGTAGGC; mCOX-2: F-GAAGTCTTTGGTCTGGTGCCTG, R-GTCTGCTGGTTTGGAATAGTTGC; miNOS F-CAGCTGGGCTGTACAAAC, R-CATTGGAAGTGAAGCGGTTCG; GAPDH: F-CATGGCCTTCCGTGTT, R-CCTGGTCCTCAGTGTAGC. We calculated the real-time PCR relative gene expression level using the delta-delta Ct method.

2.8. IHC

For the histological analysis, IHC staining was performed as described previously [16]. All colon tissue paraffin sections were deparaffinized with xylene and rehydrated using various concentrations of ethanol and water. Heat-induced antigen retrieval was performed in 10 mM sodium citrate buffer containing Tween 20 (pH 6.0) using a microwave for 10 min. To block endogenous peroxidase activity, all slides were treated with 3% H_2O_2 solution for 7 min and incubated with normal horse blocking serum for 10 min to block nonspecific antibody binding. Next, all slides were incubated with specific primary antibodies at 4 °C overnight, washed three times with TBS-T buffer, and the subsequent procedures were performed using the Universal Quick kit. 3, 3′-Diaminobenzidine solution was used to detect protein signals and counterstained with hematoxylin. After mounting, a cover slip was placed on the solution, and all images were confirmed using an optical microscope.

2.9. Statistical analysis

Results are presented as mean ± standard deviation (S.D) of all samples. Statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's *post-hoc* analysis with Graph Pad Prism software 5.0. Each experiment was repeated at least three times and yielded comparable results. Values with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Mast cell deficiency alleviates CAC symptoms in AOM/DSS-induced CAC mice

We first compared the survival rate, weight, and DAI for each group. All mice in the $WT^{(-)}$ group survived. Mice in the $WT^{(+)}$ group treated with AOM/DSS showed a final survival rate of 60%, and mast cell-deficient mice ($W/W^{V(+)}$) treated with AOM/DSS showed a higher survival rate than the $WT^{(+)}$ group (Fig. 1B). In this study, mice in the $WT^{(+)}$ group showed notable body weight loss, unlike mice in the normal $WT^{(-)}$ group, whereas the mast cell-deficient mice ($W/W^{V(+)}$) group had a higher body weight than the $WT^{(+)}$ group (Fig. 1C). We also investigated one of the indicators of CAC, DAI. As shown in Fig. 2A, after each DSS treatment, a significantly elevated DAI score was observed in both $WT^{(+)}$ and $W/W^{V(+)}$ groups. At each time point (weeks 2, 5, and 8), $WT^{(+)}$ group mice presented higher DAI scores than $W/W^{V(+)}$ group mice. This suggests that mast cell deficiency alleviates the commonly observed symptoms of colon cancer.

3.2. Mast cell deficiency attenuates AOM/DSS-induced colon length shortening and colorectal tumorigenesis

Colon shortening, a morphologic index of colitis-related severity, is observed in AOM/DSS-induced CAC mice model. Colon length in both $WT^{(+)}$ and $W/W^{V(+)}$ mice group decreased significantly after three cycles of DSS treatment compared with the $WT^{(-)}$ mice group. The colon length of $W/W^{V(+)}$ mice was markedly longer than that of the $WT^{(+)}$ mice (Fig. 2B). To examine the effects of mast cell deficiency on colorectal tumor formation, we analyzed the tumor number and size in colon tissues of each group on the final day. After three cycles of DSS treatment, colorectal tumors had formed in the latter half of the colon in both the $WT^{(+)}$ and $W/W^{V(+)}$ mice groups (tumors are marked in red arrow in Fig. 2C). The incidence of colorectal tumor was lower in the middle and distal colon tissues of the $W/W^{V(+)}$ mice group than in the $WT^{(+)}$ mice group. In addition, we examined the total tumor number and maximum tumor size in the colon tissues of all groups. As shown in Fig. 2D and E, both total tumor number and maximum tumor size were significantly attenuated in $W/W^{V(+)}$ mice compared with those in the $WT^{(+)}$ mice. $WT^{(-)}$ mice had no tumors. These observations suggest that mast cell deficiency inhibits colitis-associated colorectal tumorigenesis in AOM/DSS-induced CAC mice.

3.3. Mast cell deficiency suppresses AOM/DSS-induced histopathological changes in colon cancer

To observe the histopathological changes in crypt destruction, submucosal invasion, and tumor formation in the colon tissues of each group, H&E staining was performed. No tumors or tissue damage were observed in the $WT^{(-)}$ mice group, whereas $WT^{(+)}$ mice exhibited many severe tumor-like protrusions following AOM/DSS treatment in the observed opened colon tissues. These tumors were mostly located in the middle of the distal colon and were primarily broad-based adenomas with high-grade dysplasia and varying degrees of inflammatory cell infiltration. Inflammatory cell infiltration into the colon submucosa, crypt destruction, and epithelial cell damage in colon tissue of mice in the $WT^{(+)}$ group increased notably compared to those of mice in the $WT^{(-)}$ group. In particular, in the $WT^{(+)}$ mice group, additional capillaries developed to support growth and differentiation by supplying nutrients to the tumor. However, we confirmed that colon cancers of the $W/W^{V(+)}$ mice group were significantly restored by mast cell deficiency. The colon tissue of the $W/W^{V(+)}$ group showed dysplastic crypts with hyperchromatic nuclei and light tumors or non-tumors (Fig. 3A). Therefore, the $W/W^{V(+)}$ group showed a dramatically reduced histopathological score index compared with the $WT^{(+)}$ group (Fig. 3B).

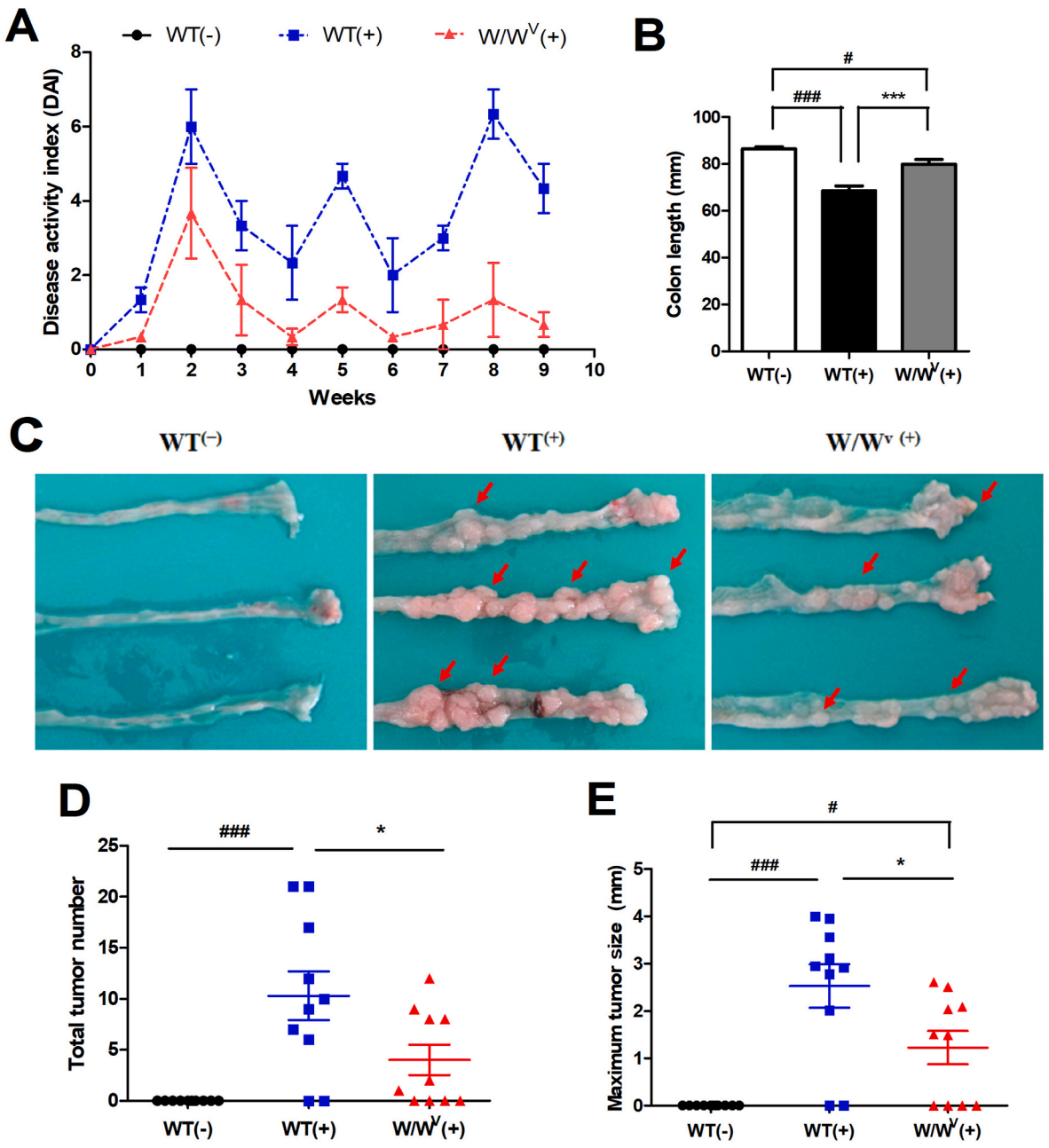

Fig. 2. Mast cell deficiency inhibits colon shortening and colorectal tumorigenesis in AOM/DSS-induced CAC mice. (A) DAI score. (B) Colon length. (C) Representative images of colon tissues (red arrow: colon cancer) of mice in each group. (D) Total tumor number and (E) maximum tumor size in the colon at day 63. All values are expressed as mean ± S. D of at least three independent experiments. Data were analyzed by Tukey's *post-hoc* analysis. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared with the WT$^{(-)}$ group; $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with the WT$^{(+)}$ group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Mast cell deficiency inhibits pro-inflammatory cytokines in AOM/DSS-induced CAC mice

We analyzed the expression levels of cytokines in the colon tissues of all mice in each group to investigate the effect of mast cell deficiency on the secretion of inflammatory mediator cytokines in AOM/DSS-induced CAC mice. As shown in Fig. 3C–E, the mRNA expression levels of pro-inflammatory cytokines in colon tissues were notably elevated in the WT$^{(+)}$ group compared with those in the WT$^{(-)}$ group. However, the expression rates of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were approximately 33.82%, 56.98%, and 26.24% lower in the W/W$^{V(+)}$ group than in the WT$^{(+)}$group, respectively.

3.5. Mast cell deficiency decreases inflammation-related signaling mRNA levels in AOM/DSS-induced CAC mice

NF-κB, COX-2, and iNOS are important signaling substances in the inflammatory response. Thus, an increase in these factors is associated with CAC. Therefore, we investigated the expression of NF-κB, COX-2, and iNOS in colon tissues. As shown in Fig. 4A, AOM/DSS treatment led to the up-regulation of inflammatory-related mRNAs, but a significant downregulation was observed in W/W$^{V(+)}$ mice by mast cell deficiency.

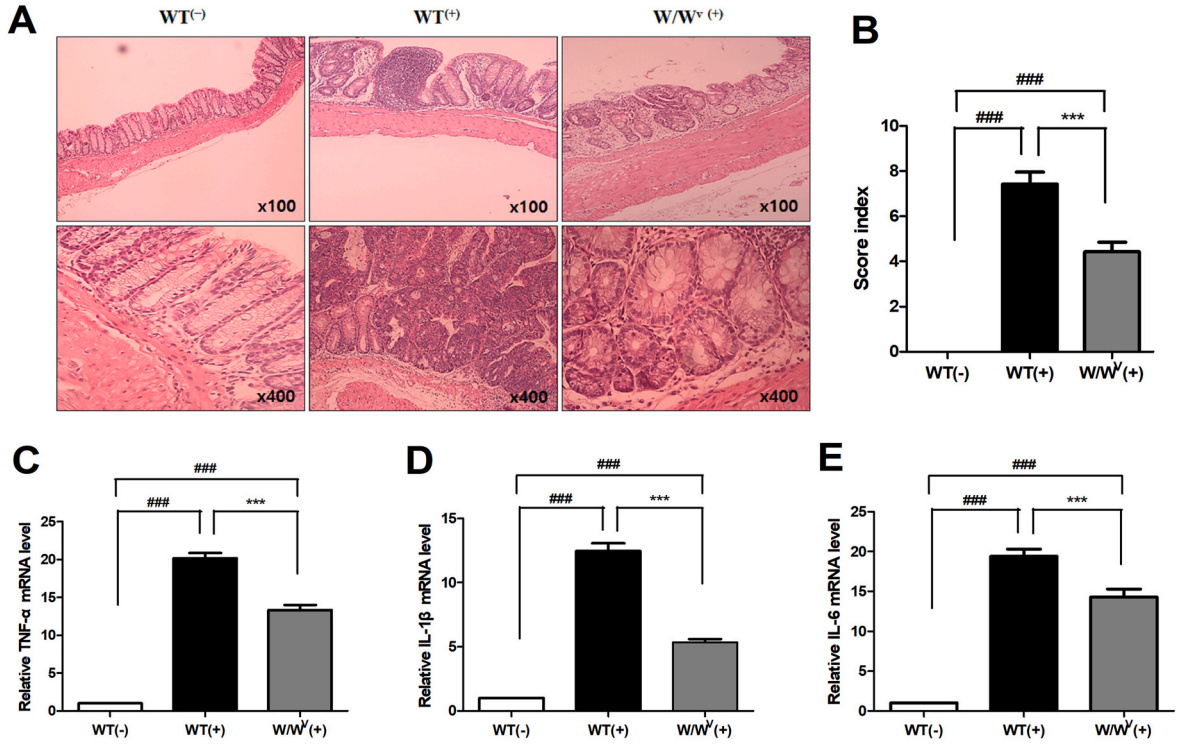

Fig. 3. Mast cell deficiency alleviates histological change and pro-inflammatory cytokine secretion in AOM/DSS-induced CAC mice. (A) Representative H&E staining colonic tumor images showing histological features of colon tissues from each mice group (magnification:×100 and × 400) and (B) histopathological score index of histological criteria. mRNA levels of (C) TNF-α, (D) IL-1β, and (E) IL-6 in colon tissues. GAPDH gene was used as an internal control. We analyzed mRNA gene expression levels using the delta-delta Ct method. All values are expressed as mean ± S. D of at least three independent experiments. Data were analyzed by Tukey's *post-hoc* analysis. $^{\#\#\#}p < 0.001$ compared with the $WT^{(-)}$ group; $^{***}p < 0.001$ compared with the $WT^{(+)}$ group.

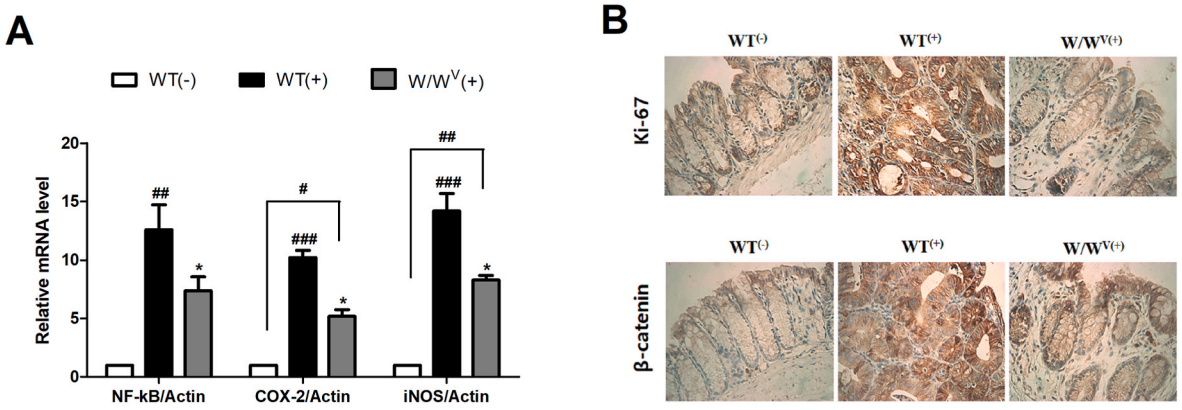

Fig. 4. Mast cell deficiency reduces the expressions of inflammation-related genes (NF-κB, COX-2, and iNOS), and tumor-related genes (Ki-67 and β-catenin) in AOM/DSS-induced CAC mice. (A) The mRNA expression of NF-κB, COX-2, and iNOS in colon tissues. GAPDH gene was used as an internal control. We analyzed mRNA gene expression levels using the delta-delta Ct method. (B) Ki-67 and β-catenin in colon tissue (magnification: × 400). All values are expressed as mean ± S. D of at least three independent experiments. Data were analyzed by Tukey's *post-hoc* analysis. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the $WT^{(-)}$ group; $^{*}p < 0.05$ compared with the $WT^{(+)}$ group.

3.6. Mast cell deficiency down-regulates Ki-67 and β-catenin protein levels in AOM/DSS-induced CAC mice

We investigated how the deficiency of mast cells in the colon tissue of the CAC mouse model affects the expression of Ki-67, a tumor cell proliferation indicator, and β-catenin, a tumorigenic gene commonly found in various cancers [17,18]. IHC staining was conducted to examine the expression of Ki-67 and β-catenin in the colon tissues of mice. As shown in Fig. 4B, the expression of Ki-67 and β-catenin in the $WT^{(+)}$ group was significantly higher than that in the $WT^{(-)}$ group but was considerably lower in the $W/W^{V(+)}$ group than that in the $WT^{(+)}$ group. These results suggest that the deficiency of mast cells suppresses the expression of genes related to tumorigenic cells, thereby reducing the progression of CAC.

4. Discussion

According to previous studies, dense accumulation and severe infiltration of mast cells is observed in colon cancer lesions; these mast cells regulate the progression of colon cancer from adenoma to carcinoma [19]. Until now, the accumulation of mast cells around cancer lesions during the onset and progression of colorectal adenoma has been

confirmed, but detailed studies on the pathways or mediators involved are insufficient.

Therefore, in this study, we investigated the role and various related mechanisms of mast cells in an AOM/DSS-induced CAC mouse model. To determine the role of mast cells, WBBF$_1$-*kit*$^{W/W-v}$ (W/W^v) mice, which are mast cell-deficient mice, were used, and mast cell–sufficient control (+/+) mice were selected as the control group.

We confirmed that AOM/DSS-induced weight decrease, colon shortening, and DAI scores, which directly indicate the severity of colon cancer, improved remarkably in the mast cell-deficient mice group. In addition, it was confirmed that the mice in the W/W$^{V(+)}$ group had significantly reduced colorectal tumors and adenoma lesions compared to mice in the WT$^{(+)}$ group. This suggests that mast cell-deficient mice are less susceptible to the incidence of colorectal cancer induced by AOM/DSS.

Histopathological analysis by H&E staining revealed a number of severe colon tumors, crypt destruction, and numerous mast cells infiltrating the tumor surrounding area in the WT$^{(+)}$ group induced by AOM/DSS. However, we confirmed that the severity of the tumor and invasion and accumulation of mast cells were weakened in the W/W$^{V(+)}$ group. The results of this study showed that mast cells play an important role in tumor development and tumorigenesis in the CAC model.

The imbalance of various cytokines is assumed to play an important role in controlling colon inflammation and developing colon cancer. According to previous studies, constant exposure to inflammatory reaction can lead to tumors [20]. Inflammation is caused by various cytokines and chemokines, and TNF-α, IL-1β, and IL-6 are the major cytokines that promote inflammation in CAC. Cytokines in the inflamed colon are responsible for the destruction of the epithelial barrier, induction of apoptosis, and secretion of subsequent inflammatory mediators from intestinal cells. Mast cells express receptors for a variety of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and thus, act as intermediates between innate and adaptive immune responses [21]. To investigate how mast cell deficiency affects cytokine expression in the CAC model, we confirmed the mRNA expression levels of pro-inflammatory cytokines in colon tissues. As a result, mRNA expression of cytokines, such as TNF-α, IL-1β, and IL-6, in colon mucosal tissue was significantly lower in the W/W$^{V(+)}$ mouse group than that in the WT$^{(+)}$ group. Hence, it was confirmed that mast cells regulate the secretion of cytokines involved in inflammation and tumor progression during colon cancer development.

NF-κB is an important transcription factor involved in the processes of chronic inflammation, tumor formation, and immune regulation and is activated by cytokines released during intestinal inflammation. Activated NF-κB regulates the secretion of pro-inflammatory cytokines and factors, such as COX-2 and iNOS, in intestinal epithelial cells, and plays an important role in inflammation, immune responses, and tumor development [22]. COX-2 is a factor involved in IBD and colitis-related colon carcinogenesis by stimulating cancer cell proliferation through the production of prostaglandin E_2 (PGE_2). Moreover, iNOS overexpression is closely related to chronic inflammatory diseases and is a factor that regulates the incidence and tumor formation of colitis-related colon cancer [23]. We observed the expression levels of NF-κB, COX-2, and iNOS in the colon mucosa of mice in the three groups. Compared to the normal group, the expression of NF-κB, COX-2, and iNOS increased in the AOM/DSS-induced WT$^{(+)}$ groups, but the expression levels decreased significantly in the W/W$^{V(+)}$ group compared to those the WT$^{(-)}$ group.

Ki-67 is a nuclear protein involved in cell proliferation and ribosomal RNA transcription and is a representative tumor cell proliferation factor for determining cancer [17]. Increased Wnt/β-catenin signaling activity is an important factor in the progression of colitis to colon cancer [24]. Indeed, the accumulation of Ki-67 and β-catenin in colon tissue is significant in colorectal tumors. Therefore, Ki-67 and β-catenin, which appear at high expression levels in colon cancer tissues, are considered to have poor prognosis in patients with colorectal cancer, indicating that inhibitors of these related signaling pathway molecules may be potential treatments for colorectal cancer. In our results, it was confirmed that the expression levels of Ki-67 and β-catenin reduced remarkably in the W/W$^{V(+)}$ group compared to those in the WT$^{(-)}$ group.

In this study, we showed that mast cells are an important regulator of the onset and progression of CAC and identified the mechanisms and mediators involved. In conclusion, it was proved that the deficiency of mast cells inhibits colitis and progression of colon cancer by reducing the inflammatory response and controlling various inflammatory mediators. Our results suggest that mast cells are potential targets for the treatment and prevention of IBD and CAC.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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