

cells. GSIs have almost the same effect on progenitor cells of the normal and regenerating crypt, which becomes critically toxic once the epithelia have been damaged. Thus caution is needed with the use of GSIs when intestinal tissue damage is present.

Although studies have revealed various extrinsic factors promoting the regeneration of the intestinal epithelia (2, 3), the intracellular mechanism mediating the regenerative process has not been fully elucidated (15). Our data show that Notch activation maintains the larger number of IECs in the immature state, thereby promoting the proliferation and supporting the rapid recovery of IECs needed to restore proper epithelial structure. Thus we identified Notch signaling as one of the main intracellular pathways mediating the organized regenerative response of the intestinal epithelia. Although we know that several ligands and receptors of the Notch pathway are expressed in the intestine (24, 26), we do not know the precise mechanism by which these ligands activate Notch receptors, in particular IECs. A recent study by Riccio et al. (22) clearly showed that both Notch1 and Notch2 function redundantly in the intestinal epithelia and that they directly regulate the cell cycle progression of crypt progenitor cells. Thus an analysis of the Notch ligand expression is needed to understand the mechanism by which these Notch receptors could be activated during epithelial regeneration and the mechanism by which such activation could be downregulated at the later stage of regeneration.

One of our surprising findings was the upregulation of PLA2G2A in Notch-activated IECs, suggesting that Notch might also modulate immune functions of IECs. PLA2G2A is usually expressed in Paneth cells, and it is known to have an antimicrobial effect (4). The loss of the continuity of the epithelial layer allows various and abundant microorganisms to invade the submucosal area, thereby promoting inflammation and further destruction of the mucosa. Thus the local secretion of PLA2G2A at the damaged mucosal area may be quite beneficial for limiting bacterial invasion and providing a proper environment for regeneration. However, previous reports have shown that PLA2G2A is also expressed by neutrophils and macrophages accumulating at the inflamed mucosa of colitis (29, 39). Consistent with this, we observed an infiltration of PLA2G2A-positive cells in the lamina propria of inflamed mucosa in UC (Fig. 9, C–F). An RT-PCR analysis of DSS-colitis showed a significant upregulation of PLA2G2A expression in the inflamed colonic mucosa (Supplemental Fig. S3). However, such an upregulation was not inhibited with LY411,575 treatment, suggesting that the expression of PLA2G2A by neutrophils or macrophages might be less dependent on Notch activation. In those cells, intracellular pathways such as NF- κ B might function to promote expression of PLA2G2A in the inflamed colonic mucosa (37). Also, our histological analysis suggested that the upregulation of PLA2G2A secretion was not a general but a partial response in Notch-activated IECs, indicating that an additional condition is required for ectopic expression of PLA2G2A.

Our microarray analysis also revealed a number of genes other than PLA2G2A that are regulated by NICD1 in IECs. Although the results did not show an upregulation of other genes specific to Paneth cells such as lysozyme or α -defensins, our quantitative RT-PCR confirmed that genes such as clusterin or spermidine/spermine N1-acetyltransferase were also

upregulated upon Notch1 activation in LS174T cells (data not shown). Trefoil factor-1 may also promote Notch-mediated tissue regeneration because it is known to be a key factor in restitution (11). The group of genes shown in the present analysis was quite distinct from the previous microarray analysis comparing GSI-treated and untreated intestinal tissues (14, 27). Because we used an in vitro IEC-based assay, the group of genes identified can be recognized as candidates of the IEC-specific target genes of Notch. However, because only a limited number of genes were analyzed (up to 10,000 annotated genes) in the present study, a further analysis including a larger group of genes may elucidate additional genes that are regulated downstream of Notch.

In conclusion, Notch signaling acts as an indispensable intracellular signaling pathway in IECs, especially during tissue regeneration. It regulates not only the differentiation, but also the proliferation of IECs, and it also regulates the immune function of IECs. We have shown for the first time that such functions of Notch are also present in the human intestine, both under normal conditions and when tissue damage has occurred. The present study provides a novel molecular basis for the advanced understanding of the regeneration process in the human intestinal epithelia, which may be utilized to establish alternative therapies for refractory ulcers caused by various intestinal diseases.

ACKNOWLEDGMENTS

The authors thank Dr. Tetsuo Sudo for providing the Hes1 antibody, Drs. Ryoichiro Kageyama and Yasuhito Yuasa for providing the plasmids, Drs. Hisao Fukushima, Kazutaka Koganei, Kenichi Sugihara, and Hiroyuki Uetake for providing the tissue samples, Drs. Hideyuki Okano and Takaaki Ito for the helpful discussion, and Dr. Hisanobu Kawamura, Dr. Michio Onizawa, and Ms. Motomi Yamazaki for technical assistance.

GRANTS

This study was supported in part by grants-in-aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research, and Creative Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology, The Japanese Ministry of Health, Labor and Welfare, The Japanese Society of Gastroenterology, The Foundation for Advancement of International Science, Research Fund of Mitsukoshi Health and Welfare Foundation, and the Research Fund of Japan Intractable Diseases Research Foundation.

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Am J Physiol Gastrointest Liver Physiol 296:850-859, 2009. First published Jan 29, 2009;
doi:10.1152/ajpgi.00071.2008

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Signaling pathway via TNF- α /NF- κ B in intestinal epithelial cells may be directly involved in colitis-associated carcinogenesis

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Submitted 13 February 2008; accepted in final form 11 January 2009

Onizawa M, Nagaishi T, Kanai T, Nagano K, Oshima S, Nemoto Y, Yoshioka A, Totsuka T, Okamoto R, Nakamura T, Sakamoto N, Tsuchiya K, Aoki K, Ohya K, Yagita H, Watanabe M. Signaling pathway via TNF- α /NF- κ B in intestinal epithelial cells may be directly involved in colitis-associated carcinogenesis. *Am J Physiol Gastrointest Liver Physiol* 296: G850–G859, 2009. First published January 29, 2009; doi:10.1152/ajpgi.00071.2008.—Treatment with anti-TNF- α MAb has been accepted as a successful maintenance therapy for patients with inflammatory bowel diseases (IBD). Moreover, it has been recently reported that blockade of TNF receptor (TNFR) 1 signaling in infiltrating hematopoietic cells may prevent the development of colitis-associated cancer (CAC). However, it remains unclear whether the TNF- α signaling in epithelial cells is involved in the development of CAC. To investigate this, we studied the effects of anti-TNF- α MAb in an animal model of CAC by administration of azoxymethane (AOM) followed by sequential dextran sodium sulfate (DSS) ingestion. We observed that the NF- κ B pathway is activated in colonic epithelia from DSS-administered mice in association with upregulation of TNFR2 rather than TNFR1. Immunoblot analysis also revealed that the TNFR2 upregulation accompanied by the NF- κ B activation is further complicated in CAC tissues induced in AOM/DSS-administered mice compared with the nontumor area. Such NF- κ B activity in the epithelial cells is significantly suppressed by the treatment of MP6-XT22, an anti-TNF- α MAb. Despite inability to reduce the severity of colitis, sequential administration of MP6-XT22 reduced the numbers and size of tumors in association with the NF- κ B inactivation. Taken together, present studies suggest that the TNFR2 signaling in intestinal epithelial cells may be directly involved in the development of CAC with persistent colitis and imply that the maintenance therapy with anti-TNF- α MAb may prevent the development of CAC in patients with long-standing IBD.

tumor necrosis factor- α ; colitis-associated cancer; intestinal epithelial cells; carcinogenesis; TNFR2

CROHN'S DISEASE (CD) and ulcerative colitis (UC) are the two major forms of inflammatory bowel diseases (IBD). Although the etiology remains unknown, increasing findings have demonstrated that the involvement of damaged epithelia and activated immune cells in the inflamed mucosa plays an important role in their pathogenesis (15, 23, 46). In addition to the problem of persistent intestinal inflammation in IBD, one of critical complications in patients with long-standing IBD over

~10 years after onset of IBD is colitis-associated cancer (CAC) (9).

It is generally observed that tumors, especially CAC, are usually infiltrated by activated immune cells, such as T cells, neutrophils, macrophages, and dendritic cells, which secrete various cytokines and chemokines (5). Recent works suggest that a major factor in the inflammatory processes involves activated NF- κ B pathway (22). Persistent NF- κ B activation in the epithelial cells has been suggested to contribute to the development of inflammation-associated cancer including CAC (12). Consistently, it has been demonstrated that activated NF- κ B is detected in most tumors (17). Therefore, persistent activation of NF- κ B of epithelial cells in response to chronic inflammation may be an important step to intestinal carcinogenesis in CAC in IBD. Consistently, a number of reports have suggested that drugs capable of inhibiting NF- κ B activation, such as 5-aminosalicylic acid, reduce the incidence of UC-related colorectal cancer (14). These studies have been approved by the Human Research Committees.

However, it still remains unknown which factors directly induce NF- κ B activation in the process of CAC. One possible mediator of NF- κ B activation in the epithelial cells of IBD patients is TNF- α , which is markedly elevated in the inflamed intestinal milieu (31). Although TNF- α has been classically considered as an anticancer agent (34), it is currently recognized that chronically elevated TNF- α in tissues may promote cancer growth, invasion, and metastasis (42). Regarding this, animal studies have demonstrated that gene deletion of TNF- α results in the suppression of skin tumor (26). In human studies, anti-TNF- α MAb (infliximab) therapy has been recently proven to be effective in the induction or maintenance of remission in patients with CD (13, 41, 44) or UC (40) refractory to the traditional therapies, such as corticosteroid and/or immunosuppressants.

In this respect, commonly used animal model of IBD-related CAC involves administration of azoxymethane (AOM) followed by repeated dextran sodium sulfate (DSS) ingestion (32). Greten and colleagues (12) demonstrated a decrease in the size of tumors with a decrease in inflammation in the similar AOM/DSS-induced CAC model with myeloid cell-specific deletion of IKK- β , which is an upstream adoptive molecule of NF- κ B. Another group had also recently demon-

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strated that TNF- α inhibition in the same animal model prevents the development of CAC via blockade of TNF receptor 1 (TNFR1) signaling in infiltrating hematopoietic cells such as neutrophils and macrophages (37). On the other hand, Greten and colleagues also demonstrated that specific deletion of IKK- β in intestinal epithelial cells led to a decrease in the numbers of tumors (initiation) albeit without a decrease in inflammation in the AOM/DSS CAC model. Although the complex background suggests that activation of NF- κ B in epithelial cells or hematopoietic cells is involved in the development of CAC, it is still unclear whether TNF- α signaling in the epithelial cells is directly involved in such suppression of carcinogenesis.

In this study, we investigated the NF- κ B activity through TNFR1 or 2 signaling specifically in colonic epithelial cells, and assessed the immediate role of anti-TNF- α MAb against epithelial cells in colitis-induced carcinogenesis using the murine AOM/DSS-induced CAC model.

MATERIALS AND METHODS

Animals

Wild-type female C57BL/6 mice (6–8 wk old) were purchased from the Japan Clea (Tokyo, Japan) and were maintained under specific pathogen-free condition. All animal experimentations were performed in accordance with institutional guidelines and were approved by the animal review board of Tokyo Medical and Dental University, Tokyo, Japan.

Cell Culture

Murine colon carcinoma-derived cell line, CT26 (50), were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum, 500 units/ml penicillin and 100 μ g/ml streptomycin (Sigma) at 37°C in 5% CO₂. Cells were seeded at a density of 5×10^5 cells/ml in six-well plates 24 h prior to the experiments with or without recombinant (r) mouse (m) TNF- α (Peprotek, London, UK) with or without blocking anti-TNF- α MAB (MP6-XT22, rat IgG1b), which was obtained from DNAX Research Institute (Palo Alto, CA) and affinity purified (1, 47).

Western Blotting

The stripped colonic epithelial samples from mice were prepared as previously described (16) for assessment of protein and/or mRNA expression. Western blotting was performed as previously described (34). Briefly, 10–100 μ g of nucleic extracts or whole protein lysates from either the stripped epithelial samples or CT26 cells were separated by 8–15% SDS-PAGE and each protein expressions were analyzed by use of the following antibodies: anti-mouse TNFR1 polyclonal antibody (PAb), anti-mouse TNFR2 PAb (R&D Systems, Minneapolis, MN), anti-phosphorylated (p)-p65 MAb at serine 536, anti-p65 PAb, anti-p-I κ B α MAb at serine 32/36, anti-I κ B α PAb, anti-p-Fas-associated death domain (FADD) PAb, anti-cleaved caspase 3 PAb (Cell Signaling Technology, Beverly, MA), anti-FADD PAb (Epitomics, Burlingame, CA), anti- β -actin MAb (Sigma), anti-upstream factor (USF)-2 PAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse IgG-HRP, anti-rabbit IgG-HRP (GE Healthcare Bio-Sciences, Piscataway, NJ) and anti-goat IgG-HRP (Santa Cruz). Signals were generated with ECL Western Blotting Detection System (GE Healthcare Bio-Sciences).

Real-time PCR. Total cellular RNA was extracted from the stripped epithelial samples described above with RNA-Bee (Tel-Test, Friendswood, TX). Five micrograms of total RNA were used as template for reverse transcription using Superscript Reverse Transcriptase kit

(Invitrogen, Carlsbad, CA). The cDNA samples were then applied for PCR with following primer pairs: TNF- α , 5'-CTA CTG GCG CTG CCA AGG CTG T-3' and 5'-GCC ATG AGG TCC ACC ACC CTG-3'; TNFR1, 5'-GGA AAG TAT GTC CAT TCT AAG AAC AA-3' and 5'-AGT CAC TCA CCA AGT AGG TTC CTT-3'; TNFR2, 5'-GAG GCC CAA GGG TCT CAG-3' and 5'-GGC TTC CGT GGG AAG AAT-3'; IL-1 β , 5'-TTG ACG GAC CCA AAA GAT-3' and 5'-GAA GCT GGA TGC TCT CAT CTG-3'; IL-6, 5'-GCT ACC AAA CTG GAT ATA ATC GGA-3' and 5'-CCA GGT AGC TAT GGT ACT CCA GAA-3'; MIP-2, 5'-AAA ATC ATC CAA AAG ATA CTG AAC AA-3' and 5'-CTT TGG TTC TTC CGT TGA GG-3'; glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5'-CTA CTG GCG CTG CCA AGG CAG T-3' and 5'-GCC ATG AGG TCC ACC ACC CTG-3'. Real time PCR was performed with QuantiTect SYBRgreen PCR kit (Qiagen, Venio, The Netherlands) using an ABI7500 real-time PCR system and 7500 system SDS software (Applied Biosystems, Foster City, CA). Data of each mRNA expression were shown as the relative amount normalized by that of G3PDH.

Induction of Acute and Chronic Colitis and Colitis-Associated Tumor

Experiment 1. Mice ($n = 3-6$) intraperitoneally (ip) administered with or without AOM 7 days in advance were treated with 3.0% DSS (molecular weight 10,000; Yokohama Kokusai Bio, Kanagawa, Japan) in drinking water for 5 days (acute phase) followed by treatment with regular water for 2–5 days (recovery phase). Colon samples were then assessed for the histology and expression of TNF- α or TNFR level at recovery phase.

Experiment 2. Mice ip administered with or without AOM 7 days in advance were treated with 3.0% DSS-containing water for 5 days followed by ip injection of control rat IgG (1 mg/mouse) mice ($n = 5$) or anti-TNF- α MAB (MP6-XT22, 1 mg/mouse) ($n = 5$) at the end of DSS treatment (*day 5*) before the removal of DSS from drinking water for 2–5 days. Colon samples were then examined for histological changes and cytokine expression profiles. Activities of NF- κ B pathway were assessed for the effect of MP6-XT22 in the development of acute colitis.

Experiment 3. To assess the role of TNF- α signaling on the incidence and progression of colon tumors in chronic AOM/DSS-induced CAC model, mice were randomized by body weight into two groups and received AOM ip at *day -7*, followed by three cycles of 3.0% DSS in drinking water for 5 days and regular water for 16 days starting 1 wk after the AOM injection (*day 0*). Mice were treated with 1 mg/mouse of either control IgG or the anti-TNF- α MAB MP6-XT22 weekly starting at the end of the first DSS treatment (*day 5*) ($n = 10$). Eleven weeks after the first injection of AOM, mice were euthanized and colons were removed, flushed with PBS, fixed as "Swiss rolls" in 10% neutral-buffered formalin at 4°C overnight, and paraffin embedded for histology.

Determination of Clinical Score of Colitis

Body weight, stool consistency, and occult or gross blood per rectum were determined every other day during the colitis induction phase. The colitis clinical score was assessed by trained individuals blinded to the treatment groups (2). The baseline clinical score was determined on *day 0*. Briefly, no weight loss was registered as 0; weight loss of 1–5% from baseline was assigned 1 point; 5–10% was assigned 2 points; 10–20% was assigned 3 points; and >20% was assigned 4 points. For stool consistency, 0 points were assigned for well-formed pellets, 2 points for pasty and semiformal stools that did not adhere to the anus, and 4 points for liquid stools that did adhere to the anus. For rectal bleeding, 0 was assigned for no blood, 2 points for positive Hemoccult, and 4 points for gross bleeding. These scores were added resulting in a total clinical score ranging from 0 (healthy) to 12 (maximal activity of colitis).

Histological Scoring of Colitis and CAC

Tissue samples were fixed in 10% neutral-buffered formalin. For the assessment of colitis, paraffin-embedded sections (5 μ m) were stained with hematoxylin and eosin (H&E). The sections were analyzed without prior knowledge of the type of treatments. The degree of inflammation in the colon was graded according to the previously described scoring system (2). Briefly, the presence of occasional inflammatory cells in the lamina propria was assigned a value of 0; increased numbers of inflammatory cells in the lamina propria a value 1, confluence of inflammatory cells, extending into the submucosa a value of 2, and transmural extension of the infiltrate a value of 3. For tissue damage, no mucosal damage was scored as 0; discrete lympho-epithelial lesions were scored as 1; surface mucosal erosion or focal ulceration was scored as 2; and extensive mucosal damage and extension into deeper structures of the bowel wall were scored as 3. The combined histological score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage). For CAC assessment, sections (5 μ m) were cut stepwise (200 μ m) through the complete block and stained with H&E. Tumor counts were performed in a blinded fashion.

Immunohistochemistry

Colon samples for immunohistochemistry were embedded into OCT compound, snap-frozen in liquid nitrogen, and stored at -80°C . Cryosections (7 μ m) were fixed in 4% paraformaldehyde for 10 min and incubated with 3% hydrogen peroxide for 15 min at room temperature. The sections were blocked with 1% BSA for 60 min and then incubated with either anti-mouse TNF- α MAb (BD Biosciences, Franklin Lakes, NJ), anti-mouse CD4 MAb (BD Biosciences), anti-mouse F4/80 MAb (eBioscience, San Diego, CA), or isotype-matched control Ig (BD Biosciences), at 4°C overnight followed by incubation with goat anti-rat Ig (Histofine Simple Stain MAX-PO, Nichirei Biosciences, Tokyo, Japan), for 30 min. The signals were visualized

by diaminobenzidine (peroxidase substrate kit, Vector Laboratories, Burlingame, CA) and the sections were counterstained with hematoxylin. For immunofluorescence studies, the sections were stained with anti- β -catenin MAb (BD Biosciences) or anti-p-p65 PAb at serine 276 (Cell Signaling Technology) followed by incubation with Alexa⁵⁹⁴-conjugated anti-mouse IgG1 or anti-rabbit IgG (Invitrogen) and DAPI (Vector Laboratory).

Statistical Analysis

Results were expressed as means \pm SE. Statistics were determined by nonparametric Mann-Whitney *U*-test, and *P* values <0.05 were considered significant.

RESULTS

TNF- α Is Markedly Upregulated in the Inflamed Mucosa of DSS-Treated Mice

To first assess whether TNF- α is induced in the inflamed colonic tissue in C57BL/6 mice by administration of DSS, mice were administered 3.0% DSS-contained drinking water for 5 days (*days 0 to 5*, acute phase) followed by removal of DSS for another 2–5 days (recovery phase) with preinjection with AOM 7 days before starting DSS treatment (Fig. 1A). Total RNA was then isolated from the colon tissues of these mice. Consistent with previous report (37), we observed significantly increased level of TNF- α mRNA at *days 7 and 10* in DSS-treated mice compared with the control preinjected with AOM without DSS treatment (Fig. 1B). This observation was also confirmed by Western blotting (data not shown). Furthermore, immunohistochemistry revealed that the majority of TNF- α -expressing cells were located in the lamina propria and

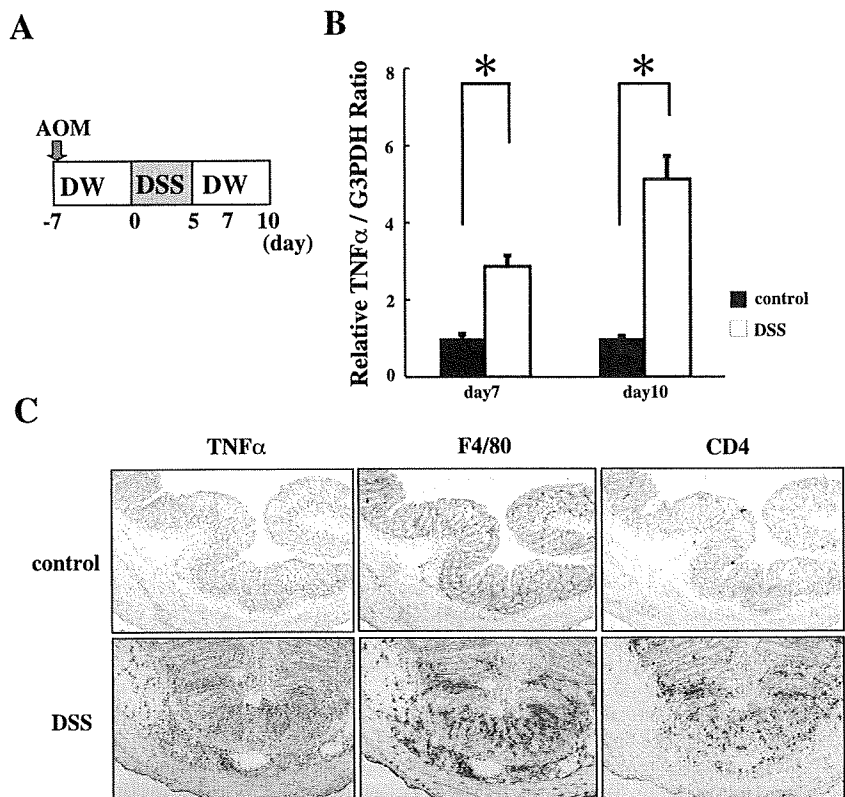


Fig. 1. TNF- α is profoundly induced in dextran sodium sulfate (DSS) colitis. **A:** protocol for acute DSS colitis. Mice were preinjected with 10 mg/kg of azoxymethane (AOM) or vehicle control 7 days before the start of DSS administration. DW, distilled water. **B:** relative mRNA expression of TNF- α in whole colon tissues that were taken from mice treated with control (regular water) or DSS at *days 7 and 10*. The levels of the mRNA were quantified by real-time PCR and normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) ($n = 3$). $*P < 0.01$. **C:** Immunohistochemistry. Frozen sections of colon tissues from control or DSS-treated mice at *day 10* were stained with anti-TNF- α , anti-CD4 or anti-F4/80 antibodies.

submucosa and some in the tunica muscularis (Fig. 1C). In addition, such TNF- α upregulation in DSS-treated colitis was not affected regardless of AOM preinjection (data not shown). Immunohistochemistry also revealed that the injured colonic tissues during the recovery phase were profoundly infiltrated by F4/80⁺ macrophages as well as CD4⁺ T cells compared with the untreated mice. Interestingly, the majority of TNF- α -expressing cells seemed to be macrophages rather than T cells (Fig. 1C). These results are consistent with the previous studies using BALB/c mice (37).

Administration of DSS Leads to Upregulation of TNFR2 but not TNFR1 in Colonic Epithelia

TNF- α -specific receptors composed of TNFR1 (p55) and 2 (p75) are known to be expressed in human intestinal epithelial cells and can be upregulated by IFN- γ (45, 49). Furthermore, it has been suggested that TNF- α is involved in the tissue repair of wounded epithelial layer (10). On the basis of the induction of TNF- α in colonic tissues by DSS treatment, TNFR1 and 2 protein expressions in colonic epithelial cells were predicted to be upregulated in this model. Therefore, we next assessed the expression levels of these molecules in the inflamed epithelial cells of AOM/DSS-treated mice. Primary colonic epithelial cells were isolated from the AOM-preinjected mice with or without DSS treatment described above at day 10 (Fig. 1A). As expected, quantitative RT-PCR (Fig. 2A) and Western blotting (Fig. 2B) showed that colonic epithelial cells expressed endogenous low amount of both TNFR1 and 2. Notably, such expression of TNFR2, but not TNFR1, was significantly upregulated by DSS treatment (Fig. 2).

NF- κ B Pathway, but not Death Domain Cascade, in Colonic Epithelial Cells Is Activated by DSS Treatment

It is suggested that the binding of TNF- α to TNFRs potentially results in the activation of two independent pathways, NF- κ B and death domain (DD) (48). Given the result of TNFR2 upregulation in DSS colitis, we next assessed the activities of NF- κ B and DD pathways in primary colonic epithelial cells from AOM/DSS-treated mice. As depicted in

Fig. 3A, I κ B α , which is upstream molecule of NF- κ B, and p65/Rel A, one of the NF- κ B components, were significantly activated in association with TNFR2 upregulation in AOM/DSS-treated colonic epithelia at day 10. In contrast, such treatment did not affect the expression of neither p-FADD nor cleaved (c)-caspase 3, a downstream molecule of DD, in these cells during the recovery phase (Fig. 3B).

TNF- α Stimulates NF- κ B Pathway in Intestinal Epithelial Cells

Given the upregulation of TNFR2 and specific activation of NF- κ B, but not DD, in colonic epithelial cells in AOM/DSS colitis, we next utilized a mouse colonic epithelial cell line, CT26, to examine whether TNFR2 signaling is associated with NF- κ B activation in these epithelial cells. CT26 cells were cultivated in the absence or presence of rIFN- γ to induce TNFRs upregulation. Although CT26 cells endogenously expressed TNFR2, its level was not upregulated in the presence of IFN- γ . This suggested that CT26 cells constitutively express maximum level of TNFR2 in the condition (data not shown). Moreover, we also observed that these cells express both p65 and I κ B α (Fig. 4A). To further confirm whether TNFR2 in those cells is functional, the activation of NF- κ B by rTNF- α was examined. Intestinal CT26 cells were stimulated with several concentrations of rTNF- α for 5 min. As shown in Fig. 4A, in vitro stimulation with rTNF- α led to the phosphorylation of p65 and I κ B α in CT26 cells in a dose-dependent fashion.

Given the upregulated expression of TNFR2 and its signal profile in the stimulated-CT26 cells, we next examined the effect of anti-TNF- α MAb on NF- κ B activation in these cells. CT26 cells were stimulated with 10 ng/ml of rTNF- α for 5 min in the presence of various concentrations of anti-TNF- α MAb, MP6-XT22. Although total expression level of p65 in rTNF- α -stimulated CT26 cells were not affected, phosphorylation of p65 in these cells was suppressed by the blockade of TNF- α with MP6-XT22 in a dose-dependent fashion (Fig. 4B). Similarly, neutralization of rTNF- α by MP6-XT22 also attenuated the phosphorylation status of I κ B α in CT26 cells in a dose-dependent fashion. Furthermore, our kinetic assessment revealed that such suppression of NF- κ B pathway by anti-TNF- α MAb did not express delayed signal response since both MP6-XT22-treated and control IgG-treated cells showed maximum level of p-p65 and p-I κ B α around 10 min after the stimulation with rTNF- α (Fig. 4C). It should be noted that we consistently observed the degradation of I κ B α 15 min after rTNF- α stimulation although the expressions of p-p65 and p-I κ B α reach maximum level at 10 min. These results indicate that TNF- α stimulation against colonic epithelial cells results in activation of NF- κ B via TNFR2 and, moreover, MP6-XT22 can arrest such activation.

Anti-TNF- α MAb Treatment Suppresses NF- κ B Activation in Acute DSS Colitis Model

On the basis of the in vitro evidence of NF- κ B inactivation by MP6-XT22 in rTNF- α -stimulated CT26 cells, we next confirmed this observation in the acute DSS colitis model to pursue more physiological function of MP6-XT22 in vivo. Because NF- κ B is known to be critical in the induction of tissue inflammation such as colitis, it was initially anticipated that treatment with anti-TNF- α MAb would suppress the de-

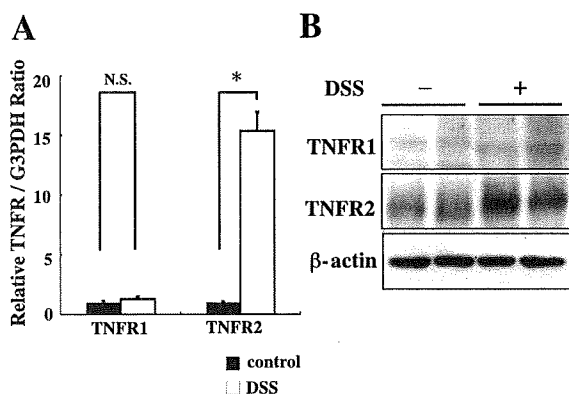


Fig. 2. TNF receptor (TNFR) 2 in colonic epithelia is upregulated in DSS colitis. A: relative mRNA expression levels of TNFR1 and 2 in colonic epithelial cells from mice treated with control (regular water) or DSS at day 10. The levels of the mRNAs were quantified by real-time PCR and normalized to the level of G3PDH ($n = 3$). * $P < 0.01$. B: protein lysates of isolated colonic epithelial cells from control- or DSS-treated mice at day 10 were subjected to Western blotting with anti-TNFR1, anti-TNFR2, or anti- β -actin antibodies.

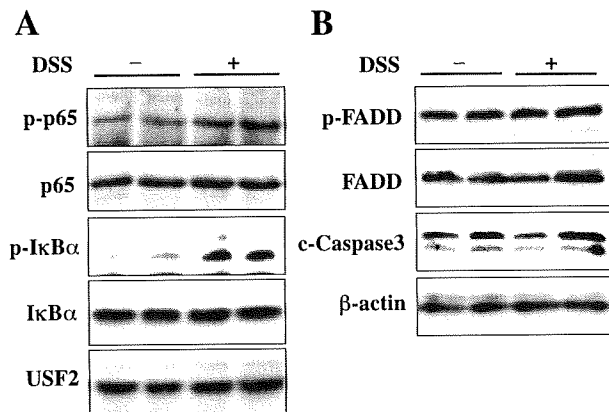


Fig. 3. NF- κ B pathway in colonic epithelial cells is activated in DSS colitis. Protein lysates of isolated colonic epithelial cells from control or DSS-treated mice at *day 10* were subjected to Western blotting with either anti-p65, anti-p-p65, anti-I κ B α , anti-p-I κ B α , or anti-USF2 antibodies for the assessment of NF- κ B activity (A) and anti-FADD, anti-p-FADD, anti-cleaved (c)-caspase 3, or anti- β -actin antibodies for apoptosis activity (B).

velopment of colitis. Therefore, we administered either control IgG or MP6-XT22 ip to the AOM/DSS-administered mice at *day 5* after DSS ingestion (Fig. 5A) followed by histological assessment at *days 7* and *10*. Then colonic epithelial cells were isolated at *day 10*. Unexpectedly, however, there was no significant difference in the histological score between two

groups irrespective of administration of anti-TNF- α MAb or control IgG (Fig. 5B). On the other hand, AOM/DSS-treated epithelial samples with control IgG showed markedly upregulated p-I κ B α and p-p65 expressions. However, injection with MP6-XT22 into AOM/DSS-treated mice resulted in suppression of I κ B α and p65 phosphorylation, although total expression levels of p65 and I κ B α were not affected in these samples (Fig. 5C). Given this discrepancy between the activities of NF- κ B and the proportions of colitis in our experiments, we subsequently evaluated cytokine production such as IL-1 β , IL-6, and MIP-2 (mouse homolog of human IL-8) in this model. Total RNA samples were extracted from colonic tissues of mice treated as above. Interestingly, quantitative RT-PCR analysis revealed significantly reduced mRNA expressions for IL-1 β , IL-6, and MIP-2 in whole colons from MP6-XT22-treated mice at *day 7* compared with those of the control group (Fig. 5D). These results indicate that induced TNF- α in mucosal tissues may not be essential for colitis exacerbation but is somehow responsible for the production of these cytokine secondary to NF- κ B activation in the DSS model.

Repetitive Anti-TNF- α MAb Treatment Suppresses Development of CAC in AOM/DSS-Treated Mice

The present protocol of anti-TNF- α MAb (MP6-XT22) administration has been useful in the assessment of TNF- α /TNFR signaling pathway on the development of CAC, because this does not affect the inflammation of DSS colitis but rather

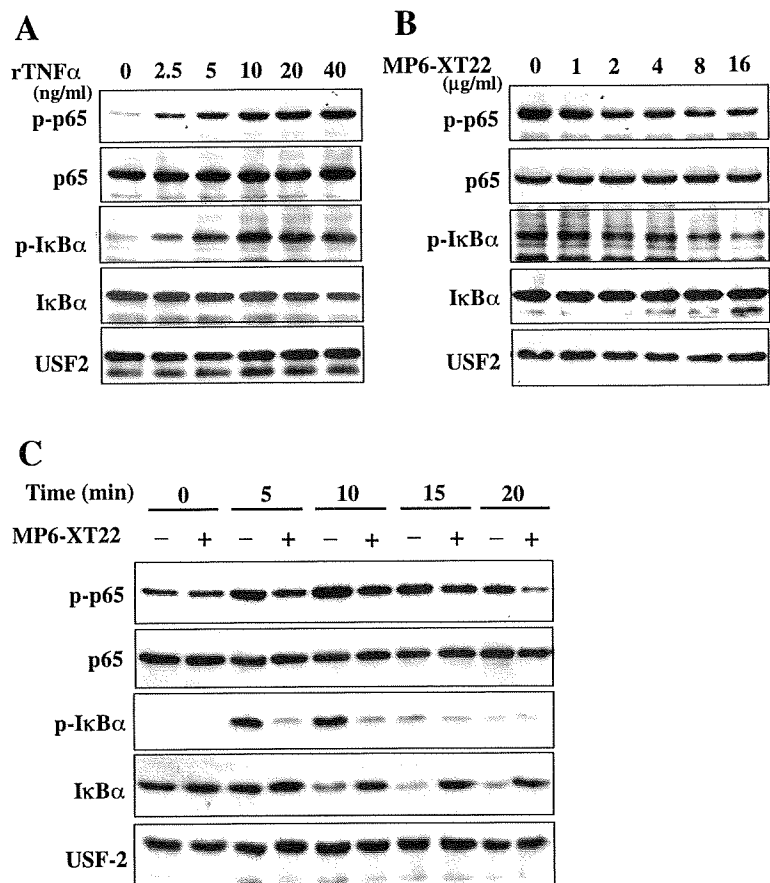


Fig. 4. MP6-XT22 suppresses NF- κ B activation in TNF- α -stimulated CT26 cells. A: protein lysates from CT26 cells stimulated with indicated concentration of rTNF- α were subjected to Western blotting with anti-p65, anti-p-p65, anti-I κ B α , anti-p-I κ B α , or anti-USF2 antibodies. Representative data at 5 min after TNF- α -stimulation are shown. B: protein lysates from 10 ng/ml rTNF- α -stimulated CT26 cells incubated with the indicated concentration of MP6-XT22 were subjected to Western blotting with anti-p65, anti-p-p65 anti-I κ B α , anti-p-I κ B α , or anti-USF2 antibodies. Representative data at 5 min after TNF- α -stimulation are shown. C: protein lysates from CT26 cells incubated with either control IgG or MP6-XT22 (10 μ g/ml) with rTNF- α (10 ng/ml) stimulation at the indicated time point were subjected to Western blotting with anti-p65, anti-p-p65 anti-I κ B α , anti-p-I κ B α , or anti-USF2 antibodies.

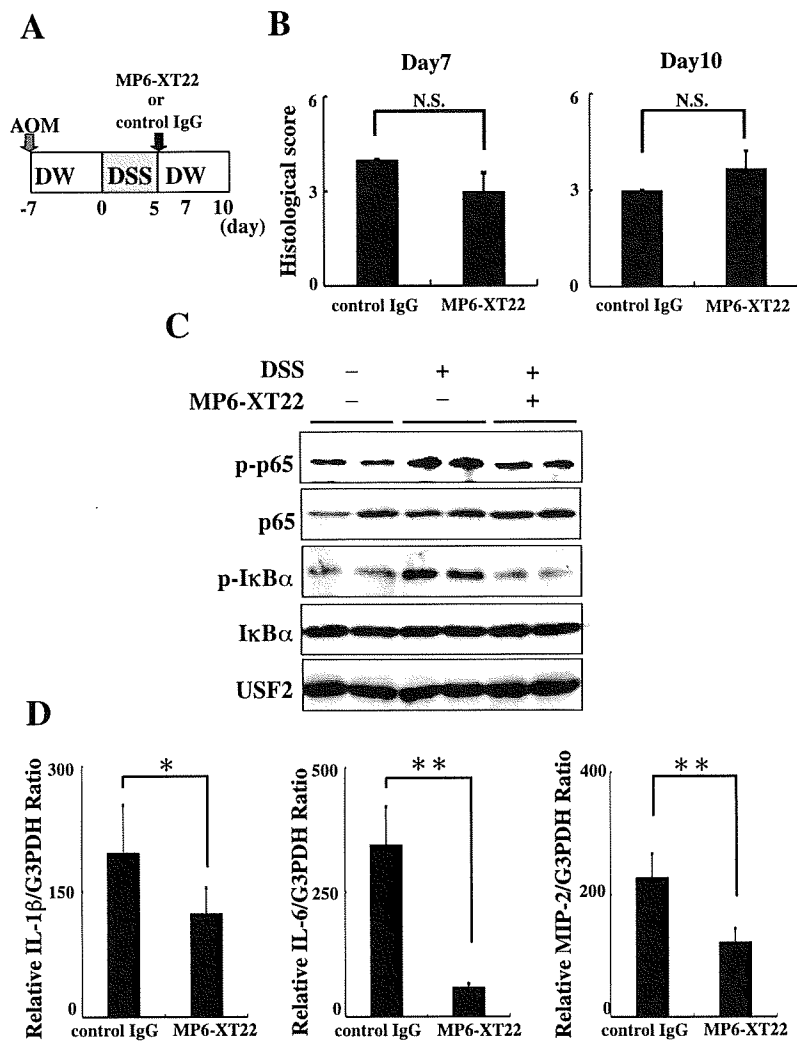


Fig. 5. MP6-XT22 treatment diminishes NF- κ B activity in DSS-treated colonic epithelial cells, but it does not ameliorate severity of colitis. *A*: protocol for acute DSS colitis. Mice received MP6-XT22 or control IgG (1 mg/mouse) at day 5. *B*: histological scores at day 7 ($n = 3$) and day 10 ($n = 6$) are shown. Data are expressed as means \pm SE. *C*: protein lysates of isolated colonic epithelial cells from control or DSS-treated mice at day 10 with or without MP6-XT22 injection were subjected to Western blotting with anti-I κ B α , anti-p-I κ B α , anti-p65, anti-p-p65, or anti-USF2 antibodies. *D*: relative mRNA expressions for IL-1 β , IL-6 and MIP-2 in whole colons from mice treated with control IgG or MP6-XT22 injection at day 10. Each mRNA level was quantified by real-time PCR and normalized to the level of G3PDH ($n = 3$). * $P < 0.01$, ** $P < 0.05$.

suppress activation of NF- κ B in epithelial cells. Therefore, this excludes the possibility that different degree of inflammation by the treatment secondarily affect the development of CAC. Thus we next investigated whether repetitive administration anti-TNF- α MAb suppresses the development of CAC in AOM/DSS-treated mice. Mice received AOM IP at day -7, followed by three cycles of DSS treatment and weekly injection of either control IgG or MP6-XT22 from day 5 until day 68. These mice were then euthanized on day 70 (see Fig. 6A). Consistent with acute model of DSS colitis as mentioned above, both groups of mice showed similar severity of clinical phenotypes as determined by weight loss, rectal bleeding, and diarrhea regardless of the treatments with MP6-XT22 (Fig. 6B). Histological findings of crypt destruction, mucosal ulceration, and infiltration were similarly observed in both groups of mice in association with the clinical scores of these mice (Fig. 6C). It should be noted that clinical and histological scores showed that the severity of inflammation was not affected by AOM treatment (data not shown).

It has been recently reported that NF- κ B activation in epithelial cells and myeloid cells plays a crucial role in carcinogenesis (12). Thus it was suggested that blockade of TNF- α

may be effective for incidence and/or progression of tumors. Therefore, we investigated the effect of anti-TNF- α MAb on the development of colitis-associated tumors in chronic DSS/AOM model. Mice administered with combination of AOM, DSS, and control IgG showed development of multiple nodular or polypoid tumors in the middle to distal colon (Fig. 7A). In contrast, decreased number of tumors was observed in mice administered with the combination of AOM, DSS, and MP6-XT22 (Fig. 7, A and B). Moreover, MP6-XT22 treatment also decreased the numbers of small tumors as well as larger tumors (Fig. 7C), despite similar morphological diversity in both groups (Fig. 7D), suggesting that the suppressed tumorigenesis in MP6-XT22-treated group is not only due to the reduced progression of tumors. Next, we thoroughly examined the histological sections, and no evidence of carcinoma such as tumor invasion into the lamina propria was observed in both control IgG and MP6-XT22 treated groups. Therefore, we defined the histology of this colitis-associated tumor model as adenoma. In addition, we also examined the β -catenin expression in these sections. The expression of β -catenin was observed in the cytoplasm, but not in the nuclei, of intestinal epithelia with colitis, as seen in Fig. 7E. However, the tumor

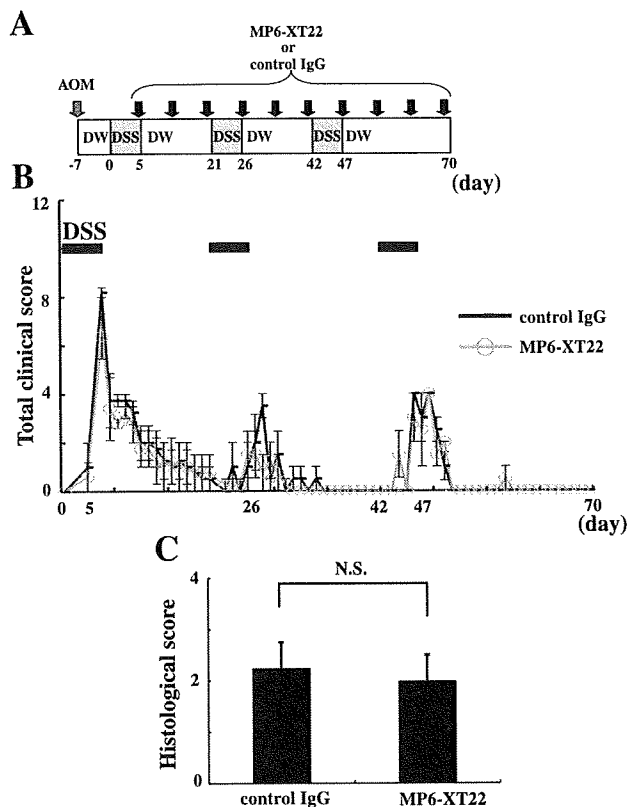
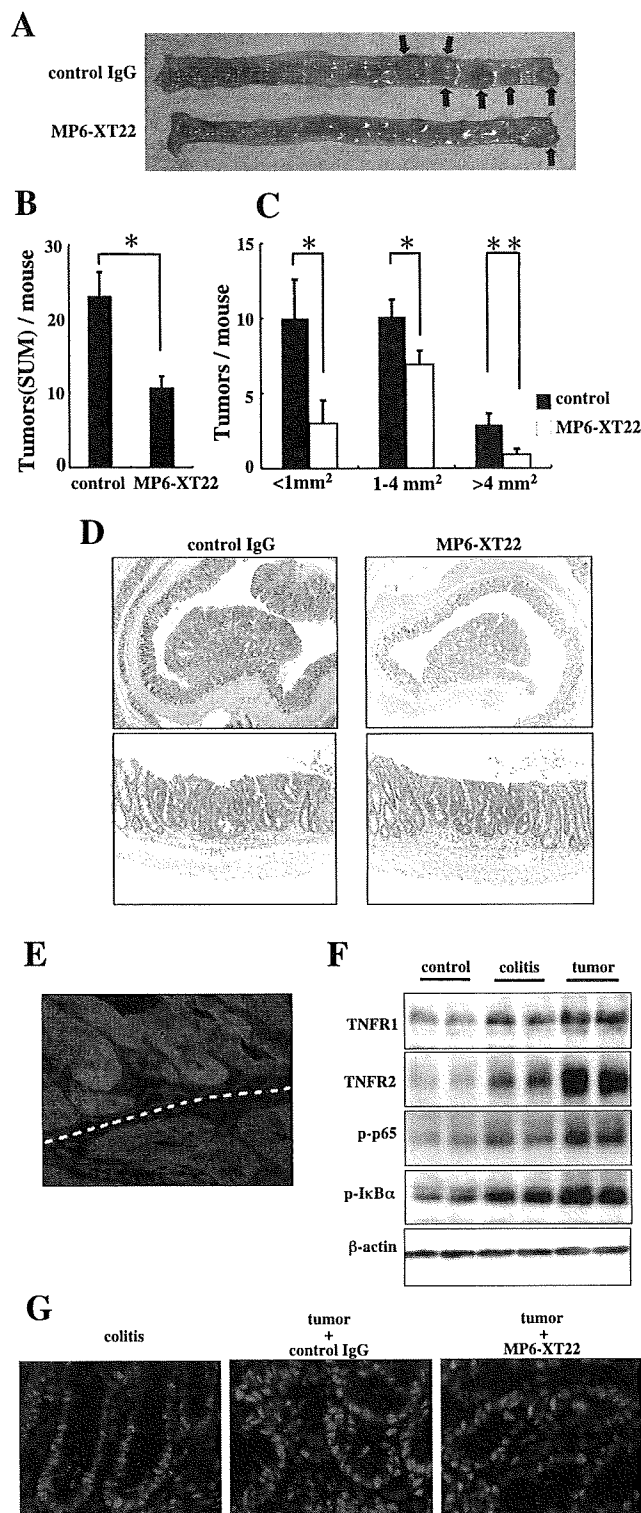


Fig. 6. MP6-XT22 does not ameliorate chronic DSS colitis. *A*: schema of the AOM/DSS colitis-associated tumor model. Mice were preinjected with AOM 7 days before starting DSS administration, and subsequently administered weekly injections of MP6-XT22 or isotype control from day 5 until day 68 during the three cycles of DSS administration. *B* and *C*: clinical (*B*) and histological (*C*) scores are shown. Data ($n = 10$) are expressed as means \pm SE. N.S., not significant.

cells showed the nuclear expression of β -catenin as well as its overexpression in the cytoplasm (Fig. 7*E*). Given the nuclear translocation of β -catenin in our tumor model (Fig. 7*E*) and the attenuation of tumor development by anti-TNF- α therapy (Fig. 7, *A*–*C*), we next examined TNFR2 expression as well as NF- κ B activities in this model. Interestingly, TNFR2 expression was more upregulated in the colitis-associated tumor. In addition, the phosphorylation of I κ B α and p65 was further induced in

Fig. 7. MP6-XT22 reduces tumor development in AOM/DSS model. *A*: macroscopic overview of representative colonic samples from each group is shown. Arrows indicate tumors. *B*: numbers of microscopically indicated tumors per colon in AOM/DSS-administered mice with isotype control or MP6-XT22 treatment ($n = 10$). Data are expressed as means \pm SE. * $P < 0.01$, ** $P < 0.05$. *C*: histogram shows tumor size distribution. Tumors were determined under the microscope. *D*: microscopic morphology of AOM/DSS tumor model. Representative histological sections with hematoxylin and eosin staining from each group are shown. *E*: β -catenin expression in colitis-associated cancer (CAC). Cryosections from AOM/DSS-treated mice were stained with anti- β -catenin MAb and anti-mouse IgG1-Alexa⁵⁹⁴. Representative sample at day 70 is shown. *Top*, tumor area; *bottom*, nontumor area. *F*: protein lysates of isolated epithelial cells from nontreated control, colitis or tumor tissues were subjected to Western blotting with anti-TNFR1, anti-TNFR2, anti-p-p65, anti-p-I κ B α or anti- β -actin antibodies. *G*: cryosections of colon tissues of healthy control, colitis, CAC with control IgG or CAC with MP6-XT22 treatment were stained with anti-p-p65 PAb and anti-rabbit IgG-Alexa⁵⁹⁴.

the tumors in association with the upregulation of TNFR2 (Fig. 7*F*). These studies indicate that the NF- κ B activation via TNFR2 in intestinal epithelial cells is closely associated with epithelial tumorigenesis. Moreover, we also observed that the nuclear expression of p65 was induced in the epithelia in tumor



tissues. However, such nuclear translocation of p65 was significantly suppressed by MP6-XT22 treatment (Fig. 7G). These findings indicate that blockade of TNF- α may contribute to suppressed initiation of colitis-associated tumors in our AOM/DSS model as well as reduction of tumor progression.

DISCUSSION

Our studies showed that blockade of TNF- α in vivo did not affect the severity of DSS-induced inflammation but rather reduced the development and progression of CAC. It has been recently reported by another group that reduced inflammation in TNFR1-deficient mice may contribute to the suppression of CAC (37). However, our results suggest that continuous suppression of TNF- α activity by MAb injection for patients with IBD, even if they are refractory to anti-TNF- α therapy, may reduce the risk of CAC.

TNF- α is a pivotal cytokine in the pathogenesis of IBD since anti-TNF- α MAb therapy is used as a powerful and promising treatment for patients with CD (13, 41, 44) or UC (40) in recent years. It has been also reported that the anti-TNF- α treatment is also effective therapy in several animal models of colitis such as TNBS colitis model, CD4⁺CD45RB^{high} T cell-reconstituted model, bone marrow transplanted tge 26 model, and Cottontop tamarin model (24, 30, 38). Interestingly, however, the effect of TNF- α MAb therapy is controversial in the DSS colitis model, which is an epithelial damage model rather than a T cell-mediated model. For example, it has been reported that anti-TNF- α antibody treatment can ameliorate acute and chronic DSS-induced colitis (20, 28). Moreover, multiple injections of anti-TNF- α Ab during the process of chronic DSS reduced the disease activity and cytokine productions in the first two cycles although adverse effects were observed in the third cycle of the DSS treatment (27). However, other groups have reported that the antibody therapy against TNF- α failed to suppress the severity of colitis in this model (20, 33). Moreover, it has been reported that DSS-induced inflammation was significantly enhanced in TNF- α deficient animals (29). This observation is somewhat similar to our results since anti-TNF- α treatment did not affect the clinical symptoms and pathological studies. Since the wounded epithelial layer by DSS treatment results in the translocation of luminal bacteria into the intestinal mucosa in this model, one of the potential interpretations regarding the different observations by others and ours is that in the setting of inflammation in acute and/or chronic DSS colitis, environmental effect such as the different microflora in different animal facilities may explain the discrepancy among the above results. Another interpretation might be that the colitis model in our studies was relatively mild compared with the others' and therefore we did not observe explicit abrogation of colitis by anti-TNF MAb administration.

Secreted TNF- α molecules in inflamed tissues can be recognized by its specific receptors such as TNFR1 and 2, which are known to be expressed in several cell types including macrophages and intestinal epithelial cells. The cytoplasmic domains of TNFR1 and 2 are known to be associated with TRAF2 by which activation of IKK is induced. Subsequently, activated IKK induces phosphorylation of I κ B, resulting in activation of NF- κ B, which consists of p50 and Rel A/p65, due to dissociation of I κ B/NF- κ B complex. In addition to NF- κ B

activation, however, TNFR1 is also capable of being recruited by FADD and TNFR-associated DD protein (TRADD) by TNF- α activation. The oligomerization of such molecules induces activation of cysteine proteases such as caspase 8, resulting in apoptosis. Interestingly, however, TNFR2, which is not coupled with FADD/TRADD complex, does not induce proapoptotic signaling when interacting with TNF- α . In this regard, we observed that TNFR2 is preferentially upregulated in regenerating epithelial cells in DSS colitis. Our interpretation of these results are that intestinal epithelial cells, when rapidly expanding, is associated with the expression of TNFR2 rather than TNFR1, and thus counter that of the proapoptotic signals of intestinal epithelia. Consistent with this, we also observed specific activation of I κ B and p65 in association with TNFR2 upregulation in wounded mucosal epithelia by DSS administration. Thus it is suggested that regenerating intestinal epithelial cells may have susceptibility of NF- κ B inactivation by blockade of TNF- α due to the specific upregulation of TNFR2 in these cells.

It is well known that long-standing UC (8, 18, 25) and CD (4, 7) are closely associated with an increased risk of CAC. To investigate the role of TNF- α signaling in colon carcinogenesis, we here used an established murine CAC model based on the mutagenic agent AOM (32, 43) along with the colitogenic administration of DSS. It has been suggested by Greten and colleagues (12) that IKK- β contributes to tumor promotion in AOM/DSS-induced CAC model. They indicated that the deletion of IKK- β in myeloid cells or epithelial cells resulted in decreased size of tumors due to reduced expression of proinflammatory cytokines that may serve as tumor growth factors (12). Moreover, it has been reported that blockade of IL-6 prevents tumor progression in AOM/DSS-induced CAC model (3). In addition, Popivanova and colleagues (37) have recently reported that the TNFR1 signaling in hematopoietic cells in colonic tissues results in production of chemokines such as CXCL1 and CCL2, which are chemotactic for neutrophils and macrophages. They also suggested that such activation of hematopoietic cells in the mucosal tissue is critical for the progression of CAC in association with suppression of DSS colitis. In our studies, colitis was mild and not clearly affected by anti-TNF- α MAb treatment despite the depressed CAC initiation and progression observed. One of the interpretations regarding different observations is that TNFR1 deficient neutrophils and macrophages on the BALB/c background may have different immunological context from that of our model on the C57BL/6 background. Some of our observation is consistent with these, because blockade of TNF- α in chronic DSS colitis by treatment with MP6-XT22 resulted in decreased cytokine expression including IL-1 β , IL-6, and MIP-2, which are considered to encourage protumorigenic activities such as angiogenesis and tumor proliferation (5, 6, 19, 21). Thus it is suggested that the blockade of TNF- α may function to suppress the secretion of these chemokines and cytokines from infiltrating lymphocytes in colitic tissue through the inhibition of NK- κ B activity in these cells. It should be noted that anti-TNF- α MAb treatment is also effective for suppression of tumorigenesis in unique mouse CAC model, which is promoted by transferring of CD4⁺CD45RB^{high} lymphocytes that may produce proinflammatory cytokine into Apc (Min/+) mice (39). This might be another evidence for the proinflammatory cytokine-induced epithelial carcinogenesis.

On the other hand, it was also suggested by Greten and colleagues (12) that deletion of IKK- β in intestinal epithelial cells led to a decrease in tumor incidence although inflammation in the tissue was not abrogated. Consistent with this, it was also suggested by another group that inactivation of NF- κ B in the hepatocytes had no effect during the course of hepatitis but failed to progress to carcinoma in an animal model of hepatitis-induced hepatocellular carcinoma (36). Although the amelioration of colitis was not apparent in our study, anti-TNF- α Ab significantly suppressed the tumor development in our AOM/DSS model in association with the downregulation of p-p65 and p-I κ B. Thus our results are consistent with observations by others above since treatment with MP6-XT22 resulted in the reduction of TNF- α -induced NF- κ B activity in colonic epithelial cells, which may influence the cell growth of colonic epithelia in DSS colitis. Regarding the involvement of apoptosis, we have tried to detect p-FADD and c-caspase 3 expressions in DSS-receiving mice, but we did not observe any significant differences compared with nontreated controls, as seen in Fig. 3B. Moreover, we have also tried to detect DNA fragmentation from rTNF- α -stimulated CT26 cells as well as TUNEL staining in the setting of recovery phase of DSS-treated or AOM/DSS-treated mice with or without anti-TNF- α treatment. However, we were not able to observe any significant difference of apoptosis activities in these experiments compared with the controls (data not shown). Therefore, we conclude that apoptosis is unlikely to be associated with the development of our colitis-associated tumor model. Moreover, we have also observed that the NF- κ B pathway is further activated in CAC tissues compared with nontumor tissues in DSS/AOM-receiving mice in association with nuclear expression of β -catenin (Fig. 7E) and extraordinary upregulation of TNFR2 (Fig. 7F). These observations evidently imply that NF- κ B activation via TNFR2 in the intestinal epithelial cells is directly correlated with the carcinogenesis induced by TNF- α stimulation. Thus it should be clarified that TNFR2 signaling in intestinal epithelia is also required for such tumor promotion as well as TNFR1 signaling in hematopoietic cells. We have therefore tried to focus on addressing this issue by using a "conventional" TNFR2-deficient model. We administered AOM and DSS treatment to these mice; however, we were not able to quantify and compare tumor development in such mice, because these mice were more sensitive to DSS colitis and had higher mortality compared with WT mice (data not shown). Our interpretation of this result is that serum TNF- α levels in conventional TNFR2-deficient mice may be elevated with LPS stimulation as previously reported by other group (35). We therefore realized that an "intestinal epithelia-specific" deficient model rather than conventional knockout mice would likely be the better model for our study. However, we were not able to obtain such conditional deficient mice. On the other hand, Fukata and colleagues (11) have recently reported that impaired Toll-like receptor-4 (TLR4) signaling reduced AOM/DSS-induced colon cancer without significant impact on inflammation. Their findings and ours may imply an important mechanism by which NF- κ B pathway is regulated in intestinal epithelial cells in setting of carcinogenesis. Thus one potential mechanism by which anti-TNF- α MAb is able to reduce the numbers and size of tumors in AOM/DSS-administered mice is that blocking TNFR2 signaling, which was presumably triggered by TLR4-mediated epithelial stimulation, in the context

of chronic intestinal wound healing may somehow decrease neoplasia even without significantly impairing inflammation. In addition, we have also observed that TNFR2 signaling in colonic epithelial cells results in the epithelial barrier dysfunction (M. Onizawa and T. Nagaishi, unpublished data). This observation suggests that TNFR2 signaling-mediated impairment of epithelial tight junction may be associated with the mechanism of tumor development.

In summary, administration of anti-TNF- α MAb directly inhibits NF- κ B activities in intestinal epithelial cells that may induce tumor formation in collaboration with independent mechanisms by another NF- κ B activity in the hematopoietic cells. Although more detail mechanism still remains to be elucidated, distinct TNF- α signaling in colonic epithelial cells may be one potential therapeutic target in the treatment of IBD-associated tumorigenesis, resulting in the change of natural history of patients with IBD.

ACKNOWLEDGMENTS

The authors thank Drs. Emiko Mizoguchi and Timothy Kuo for helpful discussions on this study.

GRANTS

This study was supported in part by grants-in-aid for Scientific Research, Scientific Research on Priority Areas, Exploratory Research and Creative Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology; the Japanese Ministry of Health, Labor and Welfare; the Japan Medical Association; Foundation for Advancement of International Science; Terumo Life Science Foundation; Ohya Health Foundation; Yakult Bio-Science Foundation; Research Fund of Mitsukoshi Health and Welfare Foundation; Japan Foundation for Applied Enzymology; Memorial Fund of Nihon University Medical Alumni Association; Abbott Japan Allergy Research Award; and Takeda Science Foundation.

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特定難治性疾患患者の回腸・大腸生検組織バンク構築

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