Prevention of Colitis-Associated Carcinogenesis with Infliximab
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Abstract
The emergence of infliximab was an epochal event in the treatment of inflammatory bowel disease (IBD). Because colitis-associated cancers arose in the setting of chronic inflammation, during which "inflammation-dysplasia-carcinoma sequence" prevails and anti-inflammatory agents can prevent carcinogenesis, we hypothesized whether infliximab can prevent colitic cancer in animal models for which C57BL/6 mice were exposed to 15 cycles of dextran sulfate sodium (DSS), with each cycle consisting of 0.7% DSS for 1 week followed by sterilized water for 10 days. Infliximab (4 mg/kg i.v.) was given on the 1st, 3rd, and 7th weeks or 25th, 27th, and 31st weeks of cycle according to "step-up" versus "top-down" strategy. Molecular change about inflammation and carcinogenesis was compared between groups. Multiple colorectal tumors developed in 75% to 80% of control mice, whereas only 16.7% of mice treated with infliximab on the 1st, 3rd, and 7th weeks developed colon tumors. Significant decreases in tumor necrosis factor-α level, mast cell number, and the expression of inflammatory cytokines were observed in top-down strategy using infliximab. The expression and activity of matrix metalloproteinase-9 (MMP-9) and MMP-11 were significantly decreased in mice treated with infliximab accompanied with attenuated numbers of β-catenin-accumulated crypts. In animal group where infliximab was administered at later stage of 25th, 27th, and 31st weeks, no reduction in tumorigenesis was noted. These biological effects of infliximab were further explored in in vitro experiment using Raw264.7 and Jurkat T cells. Conclusively, earlier and intensive therapy with infliximab should be considered for either mitigating clinical course or preventing ultimate development of colitic cancer in high-risk IBD patients. Cancer Prev Res; 3(10); OF1–20. ©2010 AACR.

Introduction
Individuals with inflammatory bowel disease (IBD) have a 10- to 40-fold increased risk of developing colorectal cancer (CRC) compared with the general population, supporting the fact that colitis-associated cancer develops from chronic persistently inflamed mucosa and progresses through dysplasia to adenocarcinoma, taking the "inflammation-dysplasia-carcinoma sequence" in contrast to "adenoma-carcinoma sequence" of sporadic CRC. Therefore, efficacious anti-inflammatory treatment can reduce or retard the development of colorectal dysplasia and cancer in IBD (1–4), and the earlier intervention of potent anti-inflammatory strategy or agents can be an effective way to rescue from colitis-associated cancer—colitic cancer (5).

The development of effective biological therapy in the treatment of IBD has given rise to a clinical debate about "step-up" versus "top-down" strategy. Step-up therapy refers to the classic therapeutic approach (i.e., progressive intensification of treatment as disease severity increases), so-called therapeutic pyramid, whereas top-down strategy refers to the early intervention of intensive therapies including biological agents or immunosuppressive agents (6–8). This concept has evolved by the emergence of infliximab, novel anti–tumor necrosis factor-α (TNF-α) biologics, which has been effective for patients who are refractory to conventional step-up treatments.

TNF-α is a prototype member of the TNF family of ligand that binds the corresponding TNF receptor family. Normally, TNF-α and other proinflammatory cytokines are maintained in balance by anti-inflammatory factors (9, 10), but this balance is shifted in favor of the proinflammatory cytokines in IBD, during which TNF-α is steeply generated by immune and even epithelial cells and contributes to tissue inflammation and destruction. The introduction of infliximab was an epoch-making event especially in the treatment of complicated Crohn’s disease such as fistula and obstruction. In addition to refractory patients to conventional diverse therapy, this agent has been shown to be effective for active IBD with rapid onset of mucosal
healing, steroid-sparing properties, inducing closure of perianal fistulas, and even maintaining remission. The therapeutic effect of infliximab is mediated by TNF-α neutralization because infliximab can bind to both soluble and transmembrane receptor (11, 12).

Based on the results of several investigators that inflammation-induced cytokines may have a pivotal role in initiation, promotion, and progression of colon carcinogenesis (13) and blocking TNF-α significantly decreased colon tumor development in a mouse model established with two-stage carcinogenesis of azoxymethane administration followed by dextran sulfate sodium (DSS; ref. 14), we hypothesized that the early interventions of infliximab can afford definite prevention of colitis cancer beyond designed mechanism of TNF-α blocking. Our study might be the first to elucidate the in vivo antitumor effect of infliximab and to emphasize the usefulness of top-down strategy for cancer-preventive purpose especially in high-risk patients presenting with longstanding and extensive involvement of IBD.

Materials and Methods

Animal model for colitis-associated cancer (colitic cancer)
Six-week-old female C57BL/6 mice (Charles River Japan) were fed sterilized commercial pellet diets (Biogenomics) and sterilized water ad libitum and housed in an air-conditioned biohazard room at a temperature of 24°C. One group composed of 24 mice. Normal control group (group 1) was administered ordinary tap water throughout the experiment, whereas disease control group (group 2) was exposed to 15 cycles of DSS (molecular weight, 40,000; ICN Pharmaceuticals) containing sterilized water, with each cycle consisting of 7 days of 0.7% DSS (w/v) in the drinking water, followed by 10 days of sterilized ordinary tap water (Fig. 1A and C). Group 3 was administered with additional daily i.p. injection of infliximab (Remicade, 4 mg/kg in 200 µL volume; Schering Pharma) as in group 2. Animals were handled in an accredited animal facility in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International Animal Care Policies and Center for Animal Care and Utilization (Gachon University of Medicine and Science). According to the interventional schedule of infliximab injection, the disease control group was set twice: once for group 3, a group treated with infliximab at the 1st, 3rd, and 7th weeks of DSS commencement, but the other for group 5, a group administered infliximab at rather later stage of 25th, 27th, and 31st weeks.

Histopathologic evaluation

Following sacrifice, the whole colons were removed. The tumors were counted on gross examination (Fig. 1A), and their sizes were measured. Tumor incidence was calculated as the number of tumor-bearing mice divided by the total number of mice, and tumor multiplicity was calculated as the number of tumors divided by the number of tumor-bearing mice. The pathology of each tumor was evaluated by two pathologists unknown to group. Each dissected colon was spread onto a plastic sheet, fixed in 10% buffered formalin for 4 hours, and prepared for paraffin tissue slides. The paraffin sections were stained with H&E. Colitis severity was graded on a scale of 0 to 3, with 0 for absence of inflammation, 1 for focal inflammatory cell infiltration, 2 for gland loss with inflammatory cell infiltrations, and 3 for ulcerations, and colitis-associated colon tumors were analyzed microscopically and diagnosed as low-grade dysplasia, high-grade dysplasia, and adenocarcinoma.

Serum and tissue levels of TNF-α

To detect the colitis activity, the levels of TNF-α in serum and colon tissues were measured in all samples. All samples were measured for their individual levels, and each sample was analyzed in triplicate manner, taking the mean of the three determinations. ELISA was applied to detect the titer of TNF-α, for which kits from R&D Systems were used.

Reverse transcription-PCR for expressions of inflammatory cytokines, real-time PCR for quantitating inflammatory cytokines, and reverse transcription-PCR for matrix metalloproteinase expressions

Total RNA was extracted using an RNeasy Mini kit (Qiagen, Inc.). Primers used for inflammatory cytokines were as follows: TNF-α, 5′-TCTCTCTGAGGACAAGACAGT-3′ and 5′-ATAGCAATTGCCCCGACG-3′; interleukin-8 (IL-8), 5′-TGGTGGGATGATGCGTCCCT-3′ and 5′-AGTGCGCGACGGCCAAGC-3′; inducible nitric oxide synthase (iNOS), 5′-GTGTTCTTCAGAGAAATGTGC-3′ and 5′-CTCTGCCCCAGTGGTCCCT-3′; cyclooxygenase-2 (COX-2), 5′-GGAGAACTCAGATGCTG-3′ and 5′-ATGAGTACGAGTACCCATAC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGTGTGACCCATCGAATGTA-3′ and 5′-TGAAGAACTGGTGCTGAGG-3′. Primers for matrix metalloproteinases (MMP), membrane-type MMPs, and tissue inhibitor of metalloproteinases (TIMP) were as follows: MMP-2, 5′-AGATCTTCTCTTCAAGCAGCCTG-3′ and 5′-GGCCGTTGCTGCTGAGGTG-3′; MMP-3, 5′-GATCGGTCAAGGAGAGGAGAGA-3′ and 5′-CTCTCCAGATCTTCTCTGAAAGA-3′; MMP-7, 5′-TACCCGACTGATAGGAAGA-3′ and 5′-AGCAACGAGGAGAGG-3′; MMP-9, 5′-GTTTGTGCTGCTGCTGCTG-3′ and 5′-GAGGAGATCGAGGACG-3′; MMP-10, 5′-ATTTGACGAGTCTCAGGATG-3′ and 5′-TGCACATATGAGAAGGAGA-3′; MMP-13, 5′-ATGAGATCTCTGAGAAGTTCTC-3′ and 5′-AGCCAGGAGGAGGAGAGA-3′; TIMP-1, 5′-ATTTCTGATTCTCTCGTGGC-3′ and 5′-CTGCTGTGCTGCTGCTGCTG-3′; TIMP-2, 5′-GCAGGAAAGCTGAGGAGAGA-3′ and 5′-CTGCTGTGCTGCTGCTGCTG-3′; TIMP-3, 5′-CTGCTGTGCTGCTGCTGCTG-3′ and 5′-CTGCTGTGCTGCTGCTGCTG-3′. Primers for MMP-9 were as follows: MMP-9, 5′-GTTTGTGCTGCTGCTGCTG-3′ and 5′-GAGGAGATCGAGGACG-3′; MMP-10, 5′-ATTTGACGAGTCTCAGGATG-3′ and 5′-TGCACATATGAGAAGGAGA-3′; TIMP-1, 5′-ATTTCTGATTCTCTCGTGGC-3′ and 5′-CTGCTGTGCTGCTGCTGCTG-3′; TIMP-2, 5′-GCAGGAAAGCTGAGGAGAGA-3′ and 5′-CTGCTGTGCTGCTGCTGCTG-3′; TIMP-3, 5′-CTGCTGTGCTGCTGCTGCTG-3′ and 5′-CTGCTGTGCTGCTGCTGCTG-3′.

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of each primer, and 2.5 units of Taq DNA polymerase (Promega) using a Perkin-Elmer GeneAmp PCR System 2400. Each cycle consists of denaturation at 95°C for 1 minute, annealing at 55°C for 45 seconds, and amplification at 72°C for 45 seconds. The reverse transcription-PCR (RT-PCR)–derived DNA fragments obtained by 25 PCR cycles were subjected to electrophoresis on a 1.5% agarose gel. The results were also scanned and quantified by densitometry. Quantitative real-time PCR was done in a LightCycler instrument (Roche Diagnostics) with the FastStart DNA Master SYBR Green I kit (Roche Diagnostics), and results were analyzed with the LDCA software supplied with the machine. Each 50-μL PCR contained 1/50th of the original cDNA synthesis reaction, 7 μL (25 mmol/L) MgCl₂, 0.8 μL (20 pmol/μL) of each primer, 1 μL (10 mmol/L) dNTP, 1 μL SYBR Green I, 0.5 μL (5 units/μL) Taq polymerase, and 5 μL of 10× buffer. Fifty cycles of amplification were done: after 94°C for 30 seconds, the annealing temperature was reduced from 94°C for 30 seconds to 57°C for 30 seconds and then to 72°C for 30 seconds. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products. Primers used for inflammatory cytokines were as follows: IFN-β, 5′-GAAGCTCTTTGTCTGTTCTGCTG-3′ and 5′-CTCTGCTTTTGGAATGTGTC-3′; iNOS, 5′-GGAGCGAGTTGAGATGTC-3′ and 5′-GTAGGGCCTTGGCTGATGAG-3′; TNF-α, 5′-GTGGAACITGCAGCAAGAGGC-3′ and 5′-AGACAGAGAGCGTTGTTGAGGC-3′.

Zymography
To detect the gelatinolytic activity in colon tissue, the samples were analyzed by gelatin. Briefly, the extracts of each mouse colon mucosa were dissolved in 2× SDS sample buffer and subjected to electrophoresis in a 10% polyacrylamide gel embedded with 0.8% acrylamide gelatin without reducing reagent. After electrophoresis, the enzymes were renatured by incubation with 3% Triton X-100, and the enzymatic reaction was allowed to proceed at 37°C for 1 hour. To determine the gelatinolytic activity, the gels were stained with 0.5% Coomassie blue in 30% methanol and 10% glacial acetic acid. Gelatin digestion was visualized as transparent bands against the dark blue background.

RNase protection assay
The RNase protection assay (RPA) is a highly sensitive and specific method for the detection and quantitation of mRNA species. Gene expressions of inflammatory cytokines and chemokines, including IL-12, IL-1β, IFN-γ, MIF, IP-10, and TCA-3 (T-cell activation-3), and gene expressions of apoptotic executors, including caspase-8, FAS, FADD (FAS-associated protein with death domain), TRAIL (TNF-related apoptosis-inducing ligand), and TRADD (TNFR1 adaptor proteins), were examined by multiprobe detection RNase protection assay (RPA)–derived DNA fragments obtained by 25 PCR cycles were subjected to electrophoresis on a 1.5% agarose gel. The results were also scanned and quantified by densitometry. Quantitative real-time PCR was done in a LightCycler instrument (Roche Diagnostics) with the FastStart DNA Master SYBR Green I kit (Roche Diagnostics), and results were analyzed with the LDCA software supplied with the machine. Each 50-μL PCR contained 1/50th of the original cDNA synthesis reaction, 7 μL (25 mmol/L) MgCl₂, 0.8 μL (20 pmol/μL) of each primer, 1 μL (10 mmol/L) dNTP, 1 μL SYBR Green I, 0.5 μL (5 units/μL) Taq polymerase, and 5 μL of 10× buffer. Fifty cycles of amplification were done: after 94°C for 30 seconds, the annealing temperature was reduced from 94°C for 30 seconds to 57°C for 30 seconds and then to 72°C for 30 seconds. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products. Primers used for inflammatory cytokines were as follows: COX-2, 5′-GAAGCTCTTTGTCTGTTCTGCTG-3′ and 5′-CTCTGCTTTTGGAATGTGTC-3′; iNOS, 5′-GGAGCGAGTTGAGATGTC-3′ and 5′-GTAGGGCCTTGGCTGATGAG-3′; TNF-α, 5′-GTGGAACITGCAGCAAGAGGC-3′ and 5′-AGACAGAGAGCGTTGTTGAGGC-3′.

Immunohistochemical stainings
After paraffin blocks were dewaxed and rehydrated with graded alcohol, these tissue sections were heated in pressure jars filled with 10 mmol/L citrate buffer using microwave for 10 minutes. After that, slides were cooled in water for 15 minutes and then washed in PBS. The slides were incubated overnight with the primary antibody. The primary antibodies were specific rabbit polyclonal antibodies against tryptase and β-catenin (Santa Cruz Biotechnology). Each antibody was diluted 1:100. After incubation, the subsequent reaction was formed using an Envision kit (DakoCytomation). Finally, the slides were incubated with 3,3′-diaminobenzidine (DakoCytomation) and counterstained with hematoxylin (Sigma). The numbers of tryptase-positive mast cells were counted on ×200 magnification field in each pathology specimen and averaged according to groups. Accumulation of β-catenin proteins, termed β-catenin–accumulated crypt (BCAC), a valuable biomarker for colon cancer bioassay, was counted by a single reader who was blinded to the treatment group. The mean numbers of BCAC noted in ×100 magnification field were counted, calculated as mean numbers, and compared among group.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining for apoptosis
Apoptosis was visualized with terminal deoxynucleotidyl transferase (TdT) FragEL DNA Fragmentation Detection kit (Oncogene Research Products). After routine deparaffinization, rehydration, and washing in 1× PBS (pH 7.4), tissues were digested with proteinase K (20 μg/mL in 1× PBS) for 20 minutes at room temperature and washed. Then, tissues were incubated in equilibration buffer for 10 minutes and treated with TdT enzyme at 37°C for 1 hour. To determine the apoptotic index (AI) in each group, we first scanned TdT-mediated dUTP nick end labeling (TUNEL)–immunostained sections under low-power magnification (×100) to locate the apoptotic hotspots. The AI at ×400 field was then scored by counting the number of TUNEL-positive cells. At least five hotspots in a section were selected, and average count was determined. Data were expressed as a mean percentage of total cell numbers.

In vitro experiment for validating the anti-inflammatory and apoptotic actions of infliximab
Raw264.7 and Jurkat T cells were cultured in culture media. As preliminary study to decide the time point when lipopolysaccharide (LPS) challenge provoked highest levels of inflammatory cytokines, the mRNA levels of TNF-α, IL-8, iNOS, and COX-2 and the protein levels of apoptosis executors were decided with RT-PCR at 2, 4, 8, 16, and
24 hours of 1 mg LPS. Raw264.7 cells showed highest induction after 4 hours, and it was 16 hours in Jurkat T cells. Next, we compared the expressions of these inflammatory mediators according to LPS alone, LPS + 1 μg infliximab (24 hours), and 1 μg infliximab alone (24 hours). These experiments were repeated according to the different doses of infliximab (1, 5, and 10 μg, respectively). Western blotting was repeated to check the changes of apoptotic executors, cytochrome c, caspase-3, caspase-9, FAS, TRADD, and poly(ADP-ribose) polymerase (PARP), according to treatment group.

**Antibodies and Western blotting**

Primary antibodies specific for cytochrome c, caspase-3, caspase-9, FAS, TRADD, and PARP were all obtained from Santa Cruz Biotechnology. Total proteins were extracted from control, LPS-treated, LPS + infliximab, and infliximab-treated cells; electrophoresed on SDS-PAGE gels; and

![Western blot diagram](image-url)

**Fig. 1.** Repeated colitis-induced tumorigenesis as animal model for colitic cancer. A and C, overview of experimental protocol of repeated DSS-induced colitic cancer model in mice (A, top-down strategy; C, step-up strategy). A, control mice were fed a normal diet and given ordinary sterilized water (group 1). Colitis was induced by 15 cycles (total, 255 d) of 0.7% DSS in drinking waters, with each cycle consisting of 7 d of 0.7% DSS, followed by 10 d of ordinary sterilized water (group 2). Group 3 was administered with infliximab at the 1st, 3rd, and 7th weeks with the same protocol as in group 2. Gross and microscopic pictures of colitic cancers. Colon tumor was observed in 9 of 12 mice of group 2 on gross inspection (left), of which pathology of each representational tumor in each mice developed in group 2 was shown (right). Magnification, ×100.
transferred to polyvinylidene difluoride membranes using a semidyry transfer system (Hoefer Pharmacia Biotech). Nonspecific binding sites were blocked by incubation with 5% nonfat dry milk in TBS-T [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% Tween 20 (v/v)] at room temperature for 1 hour. The membranes were incubated overnight at 4°C with a 1:500 dilution of primary antibody in blocking solution, followed by incubation with 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibody. The immunocomplexes were detected using an enhanced chemiluminescence detection kit (Amersham Biosciences) and autoradiographed onto X-ray film.

**Statistical analysis**

Results are expressed as the mean ± SD. The data were analyzed by one-way ANOVA, and the statistical significance between groups was determined by Duncan’s multiple range test. Statistical significance was accepted with \( P < 0.05 \).

**Results**

**Establishment of animal model for colitis-associated cancer and prevention with earlier intervention of infliximab**

Mice were randomized into five groups as follows: group 1, normal control group; group 2, repeated DSS administration group for 37 weeks; group 3, same as with group 2, but further treated with infliximab three times at 1st, 3rd, and 7th weeks i.v. (Fig. 1A); group 4, repeated DSS administration group for 37 weeks as the control group in group 5; and group 5, same as with group 4, but further treated with infliximab three times at 25th, 27th, and 31st weeks. There was no significant difference in tumor incidence between group 4 and group 5, suggesting that later intervention of infliximab was not effective in cancer prevention as noted in group 3.

Fig. 1. Continued. B, incidence of colon tumor in colitic cancer model according to group. Statistically significant decreases in tumor incidence were noted in group with early intervention of infliximab (group 3). C, overview of experimental protocol of repeated DSS-induced colitic cancer model in mice (infliximab administered based on step-up strategy). Control mice were fed a normal diet and given ordinary sterilized water (group 1). Group 4: colitis was induced by 15 cycles (total, 255 d) of 0.7% DSS in the drinking water, with each cycle consisting of 7 d of 0.7% DSS, followed by 10 d of ordinary sterilized water as in group 2. However, group 5 was different with group 3 in the intervention point of biologics because infliximab was administered at later stage of the 25th, 27th, and 31st weeks compared with the 1st, 3rd, and 7th weeks in group 3. D, incidence of colon tumor in colitic cancer model according to group. There was no significant difference in tumor incidence between group 4 and group 5, suggesting that later intervention of infliximab was not effective in cancer prevention as noted in group 3.
27th, and 31st weeks i.v. (Fig. 1C). The basic difference between group 3 and group 5 is the intervention timing of infliximab administration—the former group 3 as earlier administration and the latter group 5 as later administration of infliximab. Consequently, repeated induction of mild colitis with DSS in drinking water led to significant development of colorectal tumor after a total of 225 days (37 weeks). The tumor incidence at sacrifice was 75.0% (9 of 12) in group 2 and 80.0% (8 of 10) in group 4, respectively. The tumor multiplicity was 1.57 ± 0.5 in group 2 and 1.85 ± 0.31 in group 4. Therefore, repeated inductions of mild colitis with 0.7% DSS in drinking water (total, 15 cycles; 1 cycle is 17 days composed of 7-day administration of 0.7% DSS in drinking water followed with 10 days of sterilized tap water drinking) led to significant development of colorectal tumor, confirming the hypothesis that chronic colitis with alternating relapse and remission course is the pathway connecting between chronic inflammation and

![Graph](image)

**Fig. 2.** Changes of TNF-α cytokine essentially engaged in colitic cancer according to group. Repetitive bouts of colitis (group 2) were significantly associated with elevated serum levels of TNF-α and tissue levels of TNF-α (A) and TNF-α mRNA expressions of nontumorous colon tissue (B). These elevated expression levels of TNF-α in group 2 were significantly attenuated in group 3.
carcinogenesis under the no administration of carcinogen. Compared with other animal model of colitic cancer, in which tumorigenesis induced azoxymethane and DSS treatment, where the tumor developed mostly on anal side and spread into proximity (14), tumors developed in the whole colon with the features of mass-forming type without any prevalent locations in our model of colitic cancer (Fig. 1A). Differences in tumorigenesis came from the results of groups 3 and 5, both of which were administered the same dosing of infliximab but differed only in the timing of administration. When infliximab was administered at the 1st, 3rd, and 7th weeks after the commencement of repeated

**Fig. 2. Continued.** C, mast cells identified with tryptase immunostaining. The mean numbers of tryptase-positive mast cells were significantly elevated in colon tumor of group 2, whereas the numbers of mast cells were statistically significantly decreased in group 3, suggesting that infliximab was very effective in inactivating mast cells, one of the main sources of TNF-α. Arrows indicate the mast cells showing positive staining with tryptase antibody. D, multiprobe RPA system for inflammatory cytokines (mCK-2 and mCK-5, respectively). Compared with group 2, the expression levels of IL-12, IL-1β, MIF, MIP-1, and IL-10 were considerably decreased in group 3.
DSS cycle in group 3, colon tumors were developed only in 2 of 12 (16.7%; Fig. 1B), whereas when infliximab was administered at the 25th, 27th, and 31st weeks in group 5, colon tumors were noted in 7 of 10 (70%; Fig. 1D), suggesting that only early intervention of infliximab imposed significant cancer-preventive roles in colitis-associated tumorigenesis but no tumor-preventive effect in late intervention of infliximab. Conclusively, infliximab could play a role in efficient cancer prevention only in the case of early intervention, but late-stage administration of infliximab did not afford this benefit.

Footprint of inflammation on colitis-induced tumorigenesis and attenuated levels of inflammatory mediators with infliximab treatment

The serum level of TNF-α was markedly increased in group 2 compared with group 1 (P < 0.01), but infliximab treatment significantly decreased the serum level of TNF-α (Fig. 2A). Tissue levels of TNF-α were also significantly decreased in infliximab treatment group compared with group 2. Pooled levels of TNF-α mRNA in colon tissues from 10 nontumorigenic mucosa nearby tumor were similar between group 1 and group 2, but the mean expression of TNF-α in nontumorigenic mucosa of group 3 was significantly decreased compared with group 2 (P < 0.01; Fig. 2B). Because mast cells are one of the major sources for producing TNF-α, we counted the numbers of mast cell according to group with tryptase antibody staining. Mast cells were predominantly found in the tumors arising from chronic colitis, whereas the mean number of mast cell was significantly decreased in group 3 (P < 0.001; Fig. 2C). These results suggested that attenuated tumorigenic outcomes of group 3 were based on efficient anti-inflammatory actions of infliximab presenting with attenuated levels of TNF-α far beyond blocking of TNF-α. Next, we did multiplex RPA using RNA extracted from pooled samples of mucosal homogenates to determine whether antimetastatic effects of infliximab were related with suppressing other kinds of cytokines and chemokines beyond TNF-α blocking. As shown in Fig. 2D, migration inhibitory factor (MIF), IL-12p40, and IL-1β, cytokines reported to be principally involved in initiation or progression of ulcerative colitis, were significantly attenuated in pooled data of group 3 compared with group 2. In a similar way, the expression of macrophage inflammatory protein-1β (MIP-1β), MIP-1α, MIP-2, and TCA-3 mRNA was increased after repeated DSS administration, but down-stream strategy of infliximab significantly decreased these expressions of RANTES, MIP-1α, MIP-2, IP-10, and TCA-3 mRNA.

Increased canvas of apoptosis in colitic cancer tissues treated with infliximab

Attenuated or abolished apoptotic footprint in tumor section is a general finding observed in colitic cancer model as well as in sporadic colon cancer because deranged apoptotic activities might be fundamentally prerequisite mechanisms for tumorigenesis as for opportunity of tumor expansion (15). Using TUNEL staining, we evaluated the apoptotic activity of colitic tumor tissues and neighbor nontumorous tissues according to group. Consequently, attenuated apoptotic activities were also noted in tumor tissues of colitic cancer as seen in Fig. 3A, whereas the increased apoptotic activities were detected even in tumor tissues of group 3, signifying that infliximab might afford considerable levels of apoptotic activities in tumor tissue. Collectively, AI was significantly increased in group 3 compared with either group 1 or group 2 (P < 0.05). If we analyzed AI only in tumor tissue, the significance might be higher, but because only three tumors were found in group 3, we presented mean AI countered in whole colon comprising tumor and tumor neighbor tissue together in Fig. 3B. RPA showed that caspase-8, FAS, FADD, TNFRp55, TRAIL, TRADD, and RIP (receptor interacting protein) were increasingly expressed in group 3 compared with either group 1 or group 3 (Fig. 3C), suggesting that apoptosis induction of infliximab could be one of antitumorigenic mechanisms of top-down strategy of infliximab.

Inhibition of MMPs and attenuated formation of BCAC in colitic cancer tissue treated with infliximab

Because MMPs are known to play a core function in either inflammation propagation or carcinogenesis (16), we checked the expression and activity of MMPs according to group. Increased expressions of MMP-3, MMP-9, and MMP-13 were noted in group 2 compared with group 1 (Fig. 4A), whereas significant reduction in increased MMP activities of group 2 was noted in group 3, documented by zymography (Fig. 4B). Taken together, the antitumorigenic action of infliximab treatment was associated with attenuated expression and activity of MMP-2, MMP-9, and MMP-11. The expression of β-catenin was further evaluated by immunohistochemical staining. As shown in Fig. 4C, β-catenin was stained mostly in the membrane of coloocytes in nontumorous mucosa, whereas β-catenin was more definitely stained in the nucleus in accordance with increased expressions in the cytoplasm and membrane in transformed glands. Definitely different expression patterns of β-catenin were noted in nontumorous mucosa and cancerous mucosa (Fig. 4C, left and right). Important findings about β-catenin in colitic cancer came from Fig. 4D, so-called the changes of BCAC. Previously, the numbers of aberrant crypt foci (ACF) were counted with 5 to 10 times magnified field of microscopy after staining with methylene blue to reflect the potentiality of pre-malignant lesion. However, BCAC has been acknowledged as a more specific marker for potential carcinogenic risk than ACF because of some debates about the real significance of ACF. When we counted the mean numbers of BCAC in ×100 magnified field of microscopy according to group, statistically significant increases in mean BCAC numbers were noted in group 2. On the other hand, the mean numbers of BCAC were significantly decreased in group 3 compared with group 2 (P < 0.01; Fig. 4D), elucidating that the antitumorigenic activities of infliximab might be based on their antimetastatic mechanisms. Taken together, infliximab imposed significant levels of anti-inflammatory actions in addition to
Fig. 3. AI according to group. A, TUNEL findings in tumors of groups 2 and 3. The TUNEL-positive cells were scanty detected in tumor tissues of group 2, whereas TUNEL-positive cells were apparently increased in tumor tissues in addition to nearby nontumor tissues in group 3. B, AI according to group. AI was significantly increased in group 3 compared with group 1 or 2 ($P < 0.001$), suggesting that infliximab induced apoptosis in the tumor tissues. AI was calculated as the mean number of positive cells under ×100 magnified field of microscopic examination. C, multiprobe RPA system for apoptosis (mAPO-3). The most executive molecules engaged in apoptosis were increased in group 3 compared with group 2.
Fig. 4. MMPs and BCAC according to group. A and B, inhibitory actions of infliximab on MMP-2, MMP-9, and MT1-MMP. A, mRNA expression of each MMP according to group (pooled samples) measured with RT-PCR. MMP-3, MMP-9, and MMP-13 were apparently increased in group 2 compared with group 1. However, the expression and activity of MMP-9 and MMP-11 were significantly decreased in group 3 compared with group 2 ($P < 0.05$).
apoptosis execution and hindrance of β-catenin accumulation, by which results (i.e., significant attenuation in colitic tumor formations) were achieved.

**In vitro validation of anti-inflammatory action of infliximab**

To validate the findings noted from animal model as shown in Fig. 2, *in vitro* assay was done in macrophage and T cells. The expression of TNF-α was significantly increased after stimulation by *Escherichia coli* LPS in these cells, but the time for peak TNF-α induction differed according to target cell: 4 hours in Raw264.7 macrophage cells and 16 hours in Jurkat T cells. The reasons why we stimulated the cells with LPS rather than DSS were because DSS provoked only COX-2 expression in challenged cells and it was toxic to the cells (see Supplementary Fig. S1). Instead, colon epithelial cells were not good targets to LPS challenge (see Supplementary Fig. S2). TNF-α expressions were significantly decreased after infliximab treatment in Raw264.7 macrophage cells and Jurkat T cells (Fig. 5A). Further investigations whether infliximab could exert change in inflammatory mediators including IL-8, iNOS,
Fig. 5. In vitro documentations showing anti-inflammatory and apoptosis-inducing actions of infliximab. Using Raw264.7 macrophage cells and Jurkat T cells, LPS was stimulated for 4 h in the case of Raw264.7 cells and for 16 h in the case of Jurkat T cells based on preliminary study showing highest stimulation of inflammatory cytokines. A, RT-PCR results for inflammatory cytokines including TNF-α, IL-8, iNOS, COX-2, and GAPDH and quantitation results with real-time PCR in Raw264.7 and Jurkat T cells. Infliximab (1 μg) suppressed LPS-induced TNF-α, iNOS, and COX-2 expression in Jurkat T cells, whereas only suppressed TNF-α expression was noted in Raw264.7 macrophage cells with infliximab treatment.
Fig. 5. Continued.
and COX-2 in Raw264.7 and Jurkat T-cell lines were done. Four hours after LPS challenge in Raw264.7 cells, strong induction of inflammatory mediators such as TNF-α, IL-8, iNOS, and COX-2 was observed, but infliximab treatment attenuated TNF-α, iNOS, and COX-2 except IL-8, of which results were further documented with real-time PCR. Similar attenuated expressions of IL-8, iNOS, and COX-2, in addition to TNF-α, were also observed in Jurkat T cells.

Fig. 5. Continued. B, RT-PCR results for inflammatory cytokines including TNF-α, IL-8, iNOS, COX-2, and GAPDH and real-time PCR for quantitating mRNA. As the dose of infliximab is increased, these suppressive effects on LPS-stimulated inflammatory cytokines were more prominent in Jurkat T cells. These suppressive effects of infliximab were also prominently noted in Raw264.7 cells when the dose of infliximab was increased up to 10 μg. 1, control; 2, 1 mg LPS; 3, 1 mg LPS + 1 μg infliximab (24 h); 4, 1 mg LPS + 5 μg infliximab (24 h); 5, 1 mg LPS + 10 μg infliximab (24 h).
stimulated with 16 hours of LPS in the presence of infliximab. We extended the measurements of these inflammatory mediators after different dosings of infliximab (Fig. 5B). Infliximab showed a dose-dependent attenuation of IL-8, COX-2, and iNOS expression in addition to TNF-α. Real-time PCR clearly showed that infliximab in the presence of LPS challenge exerted decreases of COX-2, iNOS, and TNF-α expression in Raw cells and IL-8, iNOS, and TNF-α expression in Jurkat T cells in a dose-dependent manner.

**In vitro validation of apoptotic action of infliximab in tumors**

Because mucosal immune derangement such as the resistance of lamina propria T cells to apoptosis plays a key role...
Fig. 5. Continued. C, Western blotting for apoptosis executors and their densitometric analyses. Cytochrome c release was the main execution of apoptosis in Jurkat T cells treated with infliximab, whereas FAS, caspase-3, and TRADD were significantly operated in Raw264.7 cells after infliximab treatment.
role in persistence and perpetuation of inflammatory response in IBD, we hypothesized that one of antitumorigenic mechanisms of infliximab might be induction of apoptosis of engaging T cells or macrophages in IBD. As shown in Fig. 5C, increased expression of apoptosis executors, including caspase-3, a key effector caspase that degrades cellular proteins, FAS, TRADD, and PARP cleavage, was noted in Raw macrophage cells, signifying that both extrinsic and intrinsic apoptotic pathways were activated with infliximab, whereas cytochrome c release (i.e., intrinsic apoptosis pathway associated with cytochrome c release from mitochondria) was noted to be the main mechanism of infliximab on Jurkat T cells.

Discussion

The main goal of IBD treatment is the induction and maintenance of disease remission. Although the standard medical approach is guided by tracing clinical improvement with concurrent therapy (step-up approach), a new evolving concept for IBD treatment adopting earlier and aggressive intervention with anti-TNF therapy and immunosuppressive agents (top-down approach) is rising. Usually, infliximab is reserved for Crohn’s disease patients refractory to conventional therapies or complicated as fistulous or stenotic diseases, but randomized controlled trial showed that the top-down approach seems to be superior to conventional step-up strategy in inducing higher remission rates and avoiding corticosteroid side effects (8), raising the advocacy that infliximab can be preferentially used for the aggressive induction of remission to finally retard the progression of chronic inflammation to dysplasia or carcinoma (17). In this point, current study might be the first to document the rationale that top-down strategy should be preferred to high-risk patients who suffered from either pancolitis or longer duration of illness in wax and wane tendency to achieve the ultimate prevention of colitic cancer, emphasizing that top-down strategy should be considered than step-up approach to block inflammation-dysplasia-carcinoma sequence (Fig. 6).

In normal condition, TNF-α plays important roles in the development of the immune system and host protection against infectious pathogens, after which TNF-α has been called “a sentinel cytokine.” However, the role of TNF-α is quite different in pathologic condition, the generation of either inflammation or carcinogenesis in addition to dysregulated immune responses, leaving double-edged sword action of TNF-α and associated NF-κB (18–21). Mast cells are an important source of TNF-α with which they are involved in epithelial carcinogenesis (22, 23). Mucosal immune derangement, which is characterized by the resistance of lamina propria T cells to apoptosis, decreased production of anti-inflammatory cytokines such as IL-10 or transforming growth factor-β and increased production of TNF-α by local macrophages or monocytes, is reported to be the major contributors to the development of IBD (24, 25). Although a lot of the molecular alterations that are responsible for...
sporadic CRC also play a role in colitis-associated tumorigenesis, the final consequence of molecular alterations makes clear the difference between sporadic CRC and colitis-associated carcinogenesis (see Supplementary Fig. S3). For instance, the loss of APC (adenomatous polyposis coli) gene, which is a common early event in sporadic CRC, is less frequent and usually occurs very late in the colitis-associated dysplasia-carcinoma sequence. As another important alteration, p53 mutations occur late in sporadic colon carcinogenesis, whereas p53 mutations occur early and frequently detected in mucosa before the appearance of dysplasia or indefinite dysplasia in colitis patients. In contrast to sporadic CRC where dysplastic lesions or adenomatous polyps arise as one or two focal lesions in the colon, the premalignant changes have a tendency to be multifocal in colitis-associated tumorigenesis (26–31). All of these differences are due to the fact that colitis-associated cancer arises in the oncogenic setting based on chronic inflammation. Clinically, colitis-associated CRC affects younger individuals than sporadic CRC and commonly have mucinous signet ring cell pathology. All of these characteristics were identified in our colitis-associated carcinogenesis.

Chronic inflammation plays a critical role in gastrointestinal carcinogenesis. As examples, Helicobacter pylori–induced gastritis, hepatitis due to several etiologies (including >virus, toxin, and alcohol), liver fluke–induced cholangitis, Barrett's metaplasia, and IBD are well known inflammatory conditions or triggers that predispose to cancer development in gastroenterology (32). Chronic inflammation after H. pylori infection induced repopulation of the stomach with bone marrow–derived cells, and these cells subsequently progressive cancer through metaplasia and dysplasia, which provided the first molecular evidence how chronic inflammation exerted its role on tumor initiation (33). Another mechanism that is responsible for tumor initiation triggered by chronic inflammation is the excessive generation of free radicals and consequent DNA damages. Free radicals can induce a lot of alterations, including gene mutations and posttranscriptional modifications of cancer-related genes, such as p53. Activated neutrophils and macrophages are responsible for reactive oxygen species or reactive nitrogen species generation (34). A variety of proinflammatory cytokines and chemokines, such as TNF, IL-1, IL-6, IL-17, IL-12, and IL-8, are associated with link between chronic inflammation and carcinogenesis. Recent studies showed that IKK-β [inhibitor of NF-κB (IκB) kinase-β]–driven NF-κB activation pathway plays a pivotal role in tumor promotion and tumor progression (35). Activation of the classic IKK-β/NF-κB pathway causes increased transcription of genes, including mediators of inflammation (COX-2, iNOS, TNF, and IL-6), proteases, and inhibitors of apoptosis (BCL-XL, clAPs, GADD45β, BFL1, and SOD2; ref. 36). We confirmed the engagement of these molecules in colitis-associated carcinogenesis, and the cancer-preventive mechanism of infliximab might be through either ameliorating inflammatory mediators or inducing apoptosis. Because a lot of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, are secreted by activated monocytes or macrophages and these inflammatory cytokine–driven responses are significant pathogenic factors in IBD, infliximab exerted its anti-inflammatory effects by downregulation of proinflammatory cytokines via binding to transmembrane TNF receptor, a process known as reverse signaling (37, 38).

In addition to cytokines, several growth and angiogenic factors as well as matrix-degrading proteases, such as MMP-2, MMP-3, and MMP-9, are generally involved in tumorigenesis, invasion, and metastasis. MMPs can be released in response to proinflammatory cytokines such as TNF-α and IL-1β and play an important role in the process of tissue remodeling and destruction, especially MMP-9 is the most abundantly expressed protease in inflamed tissues of colon. In addition, MMPs, including MMP-2 and MMP-9, are expressed in human and animal cancers (16, 39). Increased levels of COX-2 mRNA and proteins are found in premalignant lesions as well as in malignant tissues (40). About CRC, increased expression of COX-2 was reported in 85% and 50% of human CRCs and adenomas, respectively. Infliximab imposed significant inhibition of these tumorigenic actions on colitic cancer. ACF are now frequently used as effective surrogate biomarkers of CRCs, but the preneoplastic or precancerous nature of ACF in rodents and humans still remains inconclusive (41). Instead of these obscuring implications of ACF, early-appearing BCACs have been described in en face preparations of colonic mucosa and are suggested to be premalignant in much higher fidelity than ACF. Histologic observation showed that BCACs exhibit cellular dysplasia and higher cellular proliferation and more likely to progress to malignant transformation, by which BCACs are acknowledged as an intermediate biomarker for colon carcinogenesis (42). Therefore, significant decreases in BCAC with infliximab as shown in Fig. 4D might be the significant evidence supporting cancer-preventive action of infliximab.

5-Aminosalicylic acid (5-ASA), a first-line agent for the remission induction and maintenance of mild to moderate ulcerative colitis, has been identified as a candidate chemopreventive agent. Although some meta-analyses showed that 5-ASA use can lower the risk of CRC, there remain other reports showing no protective effect against colitis-associated CRC done in a rather large case-control study (43, 44). Ikeda et al. (45) revealed that 5-ASA, given in the remission phase of colitis, significantly suppressed the development of colitis-associated cancer in a mouse model, which indicates the clinical importance of adopting chemopreventive strategies even in patients of remission state. However, the fact that we could not observe any benefit of group treated with late intervention of infliximab and 5-ASA can impose hindrance to proliferation and mutagenesis, but not so higher levels of achievement lead to conclusion that the potency seems to be enriched to achieve higher goal of colitic cancer prevention. In conclusion, although the more clear applicability of top-down approach for chemopreventive purpose should be decided with well-designed prospective clinical trials, our results drawn from animal study offered the perspective of colitic cancer prevention with top-down strategy in high-risk IBD patients presenting with extensive and easily recurred clinical course.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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