Constitutive NF-κB Activation in Colorectal Carcinoma Plays a Key Role in Angiogenesis, Promoting Tumor Growth

Kei Sakamoto,1,2 Shin Maeda,1,2 Yohko Hikiba,1 Hayato Nakagawa,2 Yoku Hayakawa,2 Wataru Shibata,1,2 Ayako Yanai,1 Keiji Ogura,2 and Masao Omata2

Abstract

Purpose: Nuclear factor-κB (NF-κB) is an important transcription factor in various biological processes. Constitutive NF-κB activation has been noted in many tumors, including colorectal cancers. However, the precise role of this activation in colorectal cancer is unclear.

Experimental Design: Constitutive NF-κB activation was evaluated in colorectal cancer tissues and cell lines. To inhibit NF-κB activation, we established cancer cells with stable knockdown of IκB kinase γ (NF-κB essential modulator), which is the regulatory subunit of the IκB kinase complex, by RNA interference. Cell growth and apoptosis were evaluated in wild-type cells (WT) and knocked-down cells (KD). Microarray and protein array analysis were also done. To determine involvement of angiogenesis, human umbilical vein endothelial cells were used. By s.c. transplantation of the cells into nude mice, tumor sizes, vascularity, and chemodrug sensitivity were analyzed.

Results: Constitutive NF-κB activation was observed in 40% of colorectal cancer tissues and 67% of cell lines. Cell proliferation was not different between WT and KD in vitro, whereas apoptosis mediated by tumor necrosis factor-α and 5-fluorouracil were increased in KD. Several angiogenic chemokines were decreased in KD. Human umbilical vein endothelial cells incubated in WT supernatant showed more branch points than in KD, suggesting that constitutive NF-κB activation was involved in angiogenesis. Subcutaneous tumor expansion was suppressed to 23% in KD, and vessels were also decreased. By 5-fluorouracil treatment, tumor expansion was suppressed to a greater extent in KD (to 6%) than in WT (to 50%).

Conclusion: NF-κB inhibition may represent a potent treatment modality in colorectal cancer, especially in cases with constitutive NF-κB activation.

Colorectal cancer is the second leading cause of cancer-related death in industrialized nations (1). The development of colorectal cancer results from the sequential accumulation of activating mutations in oncogenes, such as ras, and mutations, truncations, or deletions in the coding sequences of several tumor suppressor genes, including p53 and adenomatous polyposis coli (APC; ref. 2).

Over the last decade, there has been a great deal of progress in the development of new therapies for the treatment of colorectal cancer. The cytotoxic chemotherapy drug 5-fluorouracil (FU) was reformulated, and two new drugs, oxaliplatin and irinotecan, have been investigated as adjunctive therapies. In addition, targeted therapies, including monoclonal antibodies against vascular endothelial growth factor (VEGF; bevacizumab) and the epidermal growth factor receptor (cetuximab), are now standard treatments for metastatic colorectal carcinoma (3, 4). However, many cases show tolerance of such treatments (3, 4). Therefore, it is necessary to develop new approaches to replace or complement current therapies.

Nuclear factor-κB (NF-κB) transcription factors are key regulators of innate immune responses, inflammation, and cell survival (5), and are assembled by dimerization of two of the five subunits, p65 (RelA), c-Rel, RelB, p50, and p52/NF-κB2. Without stimulation, most NF-κB dimers are bound to specific inhibitory proteins, IκBs, in the cytoplasm. Many proinflammatory stimuli, such as tumor necrosis factor-α (TNFα), lipopolysaccharide, and various drugs, can activate NF-κB, primarily through IκB kinase (IKK)-dependent phosphorylation and degradation of the IκB inhibitory proteins. Most stimulation activates the IKK complex, which consists of two catalytic subunits, IKKα and IKKβ, and a regulatory component, IKKγ/NF-κB essential modulator (NEMO). Typically, IKK activation occurs primarily through IKKβ, the absence of which increases susceptibility to TNFα-mediated apoptosis (6). The IKK-dependent phosphorylation
Target for the treatment of colorectal cancer. The results suggest that NF-κB is an important transcriptional factor that controls various biological processes. Constitutive NF-κB activation has been noted in many tumors including colorectal cancer. However, the exact role of the activation in colorectal cancer is still unclear.

In the present study, we showed that constitutive activation of NF-κB was frequently observed in colorectal cancer. Cancer cells with stable knockdown of IκB kinase γ were established by using RNA interference and found tumor expansion was strongly suppressed in knocked-down cells and tumor vascularity was also suppressed. With the combination of 5-fluorouracil, tumor expansion was nearly stopped. We propose that NF-κB inhibition may represent a potent treatment modality in colorectal cancer, especially in cases with constitutive NF-κB activation.

Translational Relevance

There has been a great deal of progress in the development of new therapies for the treatment of patients with colorectal cancer. However, colorectal cancer is still the second leading cause of cancer-related death in industrialized nations. Therefore, it is necessary to develop new approaches to replace or complement current therapies.

Nuclear factor-κB (NF-κB) is an important transcriptional factor that controls various biological processes. Constitutive NF-κB activation has been noted in many tumors including colorectal cancer. However, the exact role of the activation in colorectal cancer is still unclear.

In the present study, we showed that constitutive activation of NF-κB was frequently observed in colorectal cancer. Cancer cells with stable knockdown of IκB kinase γ were established by using RNA interference and found tumor expansion was strongly suppressed in knocked-down cells and tumor vascularity was also suppressed. With the combination of 5-fluorouracil, tumor expansion was nearly stopped. We propose that NF-κB inhibition may represent a potent treatment modality in colorectal cancer, especially in cases with constitutive NF-κB activation.

Of IκBs can lead to ubiquitination and subsequent degradation. NF-κB, free of IκB, translocates to the nucleus, where it activates the transcription of target genes, including cytokines, chemokines, and antiapoptotic genes (7).

Many recent studies have indicated the constitutive activation of NF-κB in various malignant cells, such as lymphomas, leukemias, breast cancers, melanomas, pancreatic cancers, and colorectal cancers (8–10). Many of these reports suggested that constitutively active NF-κB is associated with harmful features of tumor cells, such as antiapoptotic effects, cell growth, and metastasis (8, 11). However, tumor cells with constitutively active NF-κB have not been studied in detail. In addition, the effects of such constitutive activation in vitro are not well understood.

As NF-κB activation contributes to antiapoptotic effects, cell growth, metastasis, and chemokine secretion, the inhibition of NF-κB activation may be useful in antitumor therapy. Indeed, there have been some reports of NF-κB inhibition as a method of tumor therapy (12), but these are as yet insufficient to consider the use of a NF-κB inhibitor as a first-line therapy for cancer.

The present study was designed to investigate the nature of constitutively active NF-κB in vitro and in vivo, using colorectal carcinoma cell lines. To inhibit NF-κB activation, we knocked down IκKγ, a key element in NF-κB activation, by small interfering RNA (siRNA), and established stable knockdown cell lines. In vivo, tumor expansion was strongly suppressed in knockdown cells and tumor vascularity was also suppressed. The results suggest that NF-κB inhibition may be an attractive target for the treatment of colorectal cancer.

Materials and Methods

Cell lines. The human colorectal carcinoma cell lines were maintained as instructed by the American Type Culture Collection. Human umbilical vein endothelial cells (HUVEC) were purchased from PromoCell and were maintained according to the manufacturer’s instructions.

Reagents. Human TNFα was purchased from R&D Systems. FU was purchased from Wako Pure Chemical Industries, Ltd. The polyclonal anti-phospho-IκBα(Ser32), anti-phospho-NF-κB-p65(Ser536), anti-phospho-Jun NH2-terminal protein kinase (INK; Thr183/Tyr185), and anti-phospho-p38 (Thr180/Tyr182) antibodies were purchased from Cell Signaling Technology. The anti–von Willebrand factor antibody was from DAKO. The other antibodies were obtained from Santa Cruz Biotechnology.

Animals. Six-week-old male BALB/c nu/nu (nude mice) were purchased from CLEA Japan, Inc. The animals were maintained under standard laboratory conditions at room temperature with relative humidity of 55% ± 5% (mean ± SD) and a 12/12-h light/dark cycle. This study was approved by the Ethics Committee of the Institute for Adult Diseases, Asahi Life Foundation. Animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute for Adult Diseases, Asahi Life Foundation, according to NIH guidelines.

Establishment of IκKγ knockdown cells. IκKγ siRNA plasmid and negative control plasmid were purchased from IMGENEX Corporation. Transfection was done using the FuGENE HD Transfection Reagent (Roche Diagnostics). To establish stable knockdown cell lines, we transfected siRNA plasmids into DLD-1 and LOVO cells, and cultured the cells in the presence of 100 μg/ml genetin.

Microarray analysis. RNA was extracted from DLD-1, LOVO, and knockdown cells using Isogen (Wako). The samples were treated with DNase for 1 h, and then purified using an RNA purification kit (Qiagen). We used the Agilent Human Whole Genome Array (Agilent Technologies) containing 60-mer DNA probes synthesized in situ in a 44-k format. Chips were scanned by Takara Bio Inc. according to the manufacturer’s hybridization protocol.

Quantitative real-time PCR, immunoblotting, and electrophoretic mobility shift assay. These procedures were conducted as described previously (13).

Cell cycle analysis by flow cytometry. The proportions of cells in G0/G1, S, and G2/M were determined by flow cytometric analysis of DNA content. Briefly, cells obtained by trypsinization were washed with PBS, and suspended in 70% ethanol. Then cells were washed again with PBS, and incubated in propidium iodide solution (50 μg/ml in PBS) for 1 h. Then cells were analyzed with fluorescence-activated cell sorting.

Chemokine array, human interleukin-8 (CXCL8), and monocyte chemotactic protein-1 (CCL2) ELISA. Culture supernatants were assayed using a Human Chemokine Antibody Array (RayBiotech, Inc.). The supernatants were assayed using a Human Chemokine Antibody Array (Qiagen) containing 60-mer DNA probes synthesized in situ in a 44-k format. Chips were scanned by Takara Bio Inc. according to the manufacturer’s hybridization protocol.

Adult Diseases, Asahi Life Foundation. Animal experiments were approved by the Ethics Committee of the Institute for Adult Diseases, Asahi Life Foundation.

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Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay. Cells were plated in chamber slides and incubated for 24 h, and then stimulated with TNFα (30 ng/ml) or FU (15 μmol/l) for 24 h. Apoptosis was analyzed using an ApoAlert DNA Fragmentation Assay kit (Takara Bio Inc.). Apoptosis in tumors was analyzed using paraffin-embedded sections as described above.

Measuring DNA fragments. Cells were plated in 12-well plates (5 × 104 cells/well), incubated for 24 h, and then stimulated with TNFα (30 ng/ml) or FU (15 μmol/l) for 24 h. The supernatants were harvested, and DNA fragments were measured using a Cell Death Detection ELISA plus kit (F. Hoffmann-La Roche Ltd.).

RNA interference. The interleukin 8 (IL-8) siRNA was purchased from Qiagen. The monocyte chemotactic protein-1 (MCP-1) siRNA was purchased from Promocell and were maintained according to the manufacturer’s instructions.

Constitutive NF-κB Activation in Colorectal Carcinoma


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Fig. 1. Detection of constitutively active NF-κB in colorectal carcinomas and establishment of IKKγ knock-down cell lines. A, colon cancer tissues were stained using an anti-p65 antibody. A representative sample of constitutively activated NF-κB (+400, left panel) and a negative sample (+400, right panel). The stained nuclei were recognized as dark brown spots, and pointed by arrows. B, detection of constitutively active NF-κB in colorectal cancer cell lines by electrophoretic mobility shift assay. Nuclear protein recovery was determined by immunoblotting with TF2D. To determine NF-κB band, supershift by anti-p65 antibody and anti-p50 antibody, and loss of the band by cold probe were confirmed. C, IKKγ was stably knocked down in DLD-1 and LOVO by siRNA. Cell lysates of each cell line were gel-separated and immunblotted with antibodies to the indicated proteins. NF-κB activation was determined in wild-type cells (WT) and knock-down cells (KD) by electrophoretic mobility shift assay. As a control, we transfected negative control plasmid to DLD-1 and LOVO (DLD-1 Emp and LOVO Emp). D, cell growth was measured by counting the number of cells. Cell cycle was analyzed by flow cytometry with propidium iodide staining.
purchased from GE Healthcare. These siRNAs were transfected with Lipofectamine 2000 (Invitrogen Life Technologies) into the cells. After 24 h of transfection, we changed the medium, cultured cells for 24 h more, and measured the concentration of IL-8 and MCP-1 by ELISA.

**In vitro angiogenesis assay.** Transformation of HUVEC was assessed using a Fibrin Gel In vitro Angiogenesis Assay Kit (Chemicon International, Inc.), in accordance with the manufacturer’s instructions. HUVECs were incubated in the test medium for 72 h, and branch points in several random views (200× magnification) were counted and the values averaged.

**In vivo angiogenesis assay.** A DIVA assay (Trevigen) was done in accordance with the manufacturer’s instructions. Briefly, angioreactors (silicone cylinders closed at one end) were filled with 20 μL of Trevigen’s basement membrane extract premixed with supernatant of DLD-1 or LOVO. They were then implanted s.c. into 6-wk-old nude mice. Fourteen days after implantation, the angioreactors were collected and processed. To quantify the invasion of endothelial cells into the angioreactors, they were labeled with FITC-lectin, and the fluorescent signals were determined.

**In vivo evaluation in human tumor xenograft models.** Nude mice were implanted s.c. with 1 × 10⁶ cells of the LOVO human colorectal carcinoma cell line, and tumor size was measured. For FU treatment, the method has been described previously (14). Briefly, nude mice were implanted s.c. with LOVO, and when tumors reached a diameter of approximately 5 mm, the animals were pair-matched into treatment

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![Graph](image_url)

**Fig. 2.** Increased TNFα-induced apoptosis and JNK activation in IKKγ knockdown cells. A, WT, Emp, and KD were incubated with TNFα (30 ng/mL) for 24 h and were analyzed by 46-diamidino-2-phenylindole (DAPI) and TUNEL staining, and percentages of apoptotic cells are shown. B, free DNA levels in the supernatant of WT, Emp, and KD after 24-h incubation with 30 ng/mL TNFα were measured. C, cells were lysed at the indicated times after TNFα treatment, and were gel-separated and immunoblotted with antibodies to the indicated proteins. D, antiapoptosis-related gene expression in WT and KD of LOVO was determined by real-time PCR. GAPDH RNA was used for normalization.
and control groups (day 1). Each group consisted of eight tumors. FU was administered i.p. at the dose of 100 mg/kg on days 1, 12, and 19. The control group received only PBS, the vehicle used for FU.

**Immunohistochemistry and in vivo TUNEL assay.** The tumors were removed and fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. For immunohistochemistry, sections were deparaffinized, rehydrated, treated with 3% H2O2 in PBS, and stained with H&E. For immunohistochemistry, sections were removed and fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. For immunohistochemistry, sections were removed and fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E.

**Table 1. Microarray analysis was done on RNA samples from DLD-1-WT, DLD-1-KD, LOVO-WT, and LOVO-KD

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NOTE: Those genes that were suppressed about 30% or less (<10^{-0.5}) at least in one KD were considered to be NF-κB-regulated genes. The GenBank accession number, the common name, the fold change are shown.

**Results**

**Constitutive activation of NF-κB in colorectal carcinoma.** To determine whether NF-κB was constitutively active in human colorectal carcinoma tissues, a tissue array was stained immunohistochemically with an anti-p65 antibody. NF-κB activation was determined when p65 nuclear staining was observed in >50% of the cancer cells in the carcinoma tissues (Fig. 1A and Supplemental Fig. S1); activation was observed in 40% (35 of 88) of colorectal carcinomas.

We also examined whether NF-κB was constitutively active in colorectal carcinoma cell lines by electrophoretic mobility shift assay (Fig. 1B). Constitutive NF-κB activation was observed in 6 of 9 cell lines (DLD-1, HCT15, HCT116, HT29, LOVO, and SW620), among which DLD-1 and LOVO showed relatively strong activity. Total p65 levels were slightly different among cells, but were not correlated with the levels of NF-κB activity (Supplemental Fig. S2). These results suggest that constitutive activation of NF-κB occurs frequently in colorectal carcinoma.

To explore the role of constitutive NF-κB activation in colorectal carcinomas, we established cancer cells with stable knock-down of IKKγ (NEMO), a regulatory subunit of the IKK complex, using siRNA, in DLD-1 and LOVO cells. As a control, we transfected negative control plasmid to DLD-1 and LOVO (DLD-1 Emp and LOVO Emp). IKKγ was successfully knocked down, but there were no differences in IKKα or IKKβ among wild-type cells (WT), Emp, and knock-down cells (KD; Fig. 1C). Phosphorylated p65 levels were decreased in KD as compared with WT and Emp, whereas p65 levels were unchanged.
(Fig. 1C). We also examined NF-κB activation by electrophoretic mobility shift assay, and binding activities were markedly decreased in KD (Fig. 1C).

To evaluate the effects of constitutive NF-κB activation on cell growth, we measured cell numbers at several time points and showed that cell growth did not differ among WT, Emp, and KD in either DLD-1 or LOVO (Fig. 1D). Cell-cycle of WT and KD was determined by flow cytometry, and G2/M ratio did not change in WT versus KD (Fig. 1D). Cell cycle regulators in WT and KD were also analyzed; the levels of cyclin A, cyclin D1, and cyclin E were not different between WT and KD (data not shown).

Increased TNFα-induced apoptosis and JNK activation in IKK-γ knock-down cells. It has been reported that blocking NF-κB signals reduces survival ability against TNFα stimulation. We tested TNFα-induced apoptosis in WT, Emp, and KD. Cells were incubated for 24 hours with or without TNFα (30 ng/mL), and TUNEL assay was then done. TNFα induced more apoptosis in KD than in WT in both DLD-1 and LOVO (Fig. 2A and Supplemental Fig. S3). The incubated supernatant was sampled to determine the free DNA levels as an indicator of apoptosis by ELISA. The free DNA level was increased to a greater extent in KD than in WT by TNFα treatment (Fig. 2B).

It has been suggested that NF-κB activation may prevent TNFα-induced apoptosis by attenuating the activation of JNK (15, 16). TNFα-induced JNK activation was weak in WT and Emp, whereas the activity was strongly induced in KD. These results indicate that one of the mechanisms by which constitutive NF-κB activation prevents TNFα-induced apoptosis in WT and Emp is through attenuation of JNK activation. Activation of p38 was also weak in WT and Emp as compared with KD. On the other hand, IκBα was constitutively activated with or without TNFα stimulation in WT and Emp, whereas constitutive activation was suppressed in KD, as expected (Fig. 2C).

Chemokines were secreted from cells with constitutively active NF-κB. To investigate the target genes of constitutively active NF-κB in DLD-1 and LOVO cells, transcription profiles were determined by microarray analysis. In DLD-1 cells, 234 of 43,376 genes were down-regulated in KD as compared with WT. In LOVO cells, 693 of 43,376 genes were down-regulated. The down-regulated genes included those for immune responses, antiapoptosis, and signal transduction; representative genes are shown in Table 1. We did real-time PCR analysis of the genes related to antiapoptotic effects. The levels of A20 and cIAP2 RNA expression were decreased in LOVO-KD in accordance with the results of microarray analysis, whereas CYLD and cIAP1 expression were unchanged (Fig. 2D). Several of the genes that were down-regulated were chemokines; they were strongly suppressed by inhibition of NF-κB activity. Thus, we next analyzed chemokines secreted into the supernatant of WT, Emp, and KD using a chemokine protein array. IL-8 and Gro in DLD-1 cells, and IL-8, MCP-1, Gro, and Gro in LOVO cells were highly secreted in WT and Emp relative to KD cell lines (Fig. 3A). These results suggest that human colorectal carcinomas with constitutively active NF-κB secrete such chemokines. Among these chemokines, we measured IL-8 and MCP-1 in 20 advanced cancer tissue samples and surrounding normal tissue samples. Seven tumors showed high levels of IL-8 (>500 pg/mg) and six showed high levels of MCP-1 (>80 pg/mg). Interestingly, the tissues showing high levels of IL-8 coincided almost completely with those showing elevation of MCP-1. None of the normal tissues showed high levels of IL-8 or MCP-1 (Fig. 3B).

Next, IL-8 and MCP-1 were measured in supernatants of colorectal carcinoma cell lines. High levels of IL-8 (>100 pg/mL) were detected in 4 of 9 samples, and high levels of MCP-1 (>8 pg/mL) were detected in 4 of 9 samples. Especially, DLD-1, LOVO, and SW620 secreted high IL-8 and high MCP-1, and all of the cell lines with high IL-8 showed constitutive NF-κB activation in Fig. 1B (Fig. 3C). These results suggested that constitutive IL-8 and MCP-1 secretion are strongly related to constitutive NF-κB activation. As in the case of NF-κB activation, IL-8 secretion in the supernatant of KD was markedly suppressed relative to WT and Emp especially in LOVO (Fig. 3D). MCP-1 secretion was also strongly suppressed in KD of LOVO and decreased to about 62% in KD of DLD-1 (Fig. 3D).

Angiogenic effects of tumor cells with constitutively active NF-κB. As previous studies have indicated that chemokines, including IL-8 and MCP-1, are angiogenic factors (17, 18), we evaluated the angiogenic effects of the supernatants of WT and KD. HUVECs were incubated for 72 hours in the supernatants of WT and KD. Although many branch points were formed in HUVECs incubated in WT supernatant, HUVECs incubated in KD supernatant showed fewer branch points (Fig. 4A). These results indicated that certain factors derived from cells with constitutively activated NF-κB induced angiogenic effects in HUVECs.

Next, to evaluate whether the angiogenic effect was dependent on IL-8 and/or MCP-1, WT were knocked down in IL-8 and/or MCP-1 by siRNA, and HUVECs were incubated in the supernatants of these cells. Branch points in HUVECs incubated in each supernatant were counted. Although the angiogenic effect of KD supernatant showed a marked reduction relative to WT and Emp, HUVECs incubated in KD supernatant showed fewer branch points (Fig. 4A). These results indicated that certain factors derived from cells with constitutively activated NF-κB induced angiogenic effects in HUVECs.

To confirm these observations in vivo, semiclosed silicone cylinders (angioreactors) with supernatants of WT and KD were implanted s.c. into mice for 14 days. This assay enabled comparison of the potencies of angiogenic factors or inhibitors. As expected, the KD supernatant induced markedly fewer blood vessels than that of WT (Fig. 4D).

Involvement of constitutively active NF-κB in tumor growth in vivo. To investigate the role of constitutively active NF-κB in tumor growth in vivo, 1.0 x 10^6 LOVO-WT and LOVO-KD cells were implanted s.c. into nude mice and the sizes of the resulting tumors were measured. The volumes of LOVO-KD tumors were markedly smaller than those of LOVO-WT tumors (25% on day 52; Fig. 5A). Each tumor was stained immunohistochemically with an antibody to von Willebrand factor, a marker of vascular endothelial cells. The numbers of blood vessels in LOVO-KD tumors were markedly lower than those in WT tumors (Fig. 5B). In addition, LOVO-WT tumors contained more proliferating cells, as determined by proliferating cell nuclear antigen staining, than LOVO-KD tumors (Fig. 5B), whereas both cells showed the same proliferative capacity in vitro (Fig. 1D). We also detected apoptosis by the TUNEL assay and showed that LOVO-KD tumors contained more apoptotic cells than LOVO-WT tumors (Fig. 5B). We confirmed
Fig. 3. Chemokines were secreted from cells with constitutively active NF-κB. A, chemokines secreted into the supernatant of WT and KD were analyzed using a protein array. Red, IL-8; blue, MCP-1; green, Gro; brown, Gro. &. IL-8 levels in human colorectal carcinomas and normal tissue surrounding the tumor were measured by IL-8 ELISA (*, 19,076 pg/mg; **, 12,593 pg/mg; ***, 4,656 pg/mg). MCP-1 levels were measured in the same samples as IL-8 ELISA by MCP-1 ELISA (†, 345.4 pg/mg; ††, 200.4 pg/mg; †††, 399.1 pg/mL). B, IL-8 levels in the supernatants of colorectal cancer cell lines were measured by IL-8 ELISA (*, 823.4 pg/mL; ***, 8,757.5 pg/mL). MCP-1 levels were measured in the same samples as IL-8 ELISA by MCP-1 ELISA (†, 115.7 pg/mL). C, IL-8 and MCP-1 levels in the supernatants of DLD-1-WT, DLD-1-Emp, DLD-1-KD, LOVO-WT, LOVO-Emp, and LOVO-KD.
that LOVO-WT tumor cells showed constitutive NF-κB activation by p65 nuclear staining and phosphorylated p65, whereas nuclei of LOVO-KD tumor cells showed less staining (Fig. 5B and Supplemental Fig. S4).

**Decreased sensitivity to FU treatment in tumor cells with constitutive NF-κB activation.** FU has been a mainstay of chemotherapy for colorectal cancer. To investigate the role of constitutive NF-κB activation in FU treatment, LOVO-WT, LOVO-Emp, and LOVO-KD were treated with FU and apoptotic cell death was evaluated using the TUNEL assay. As in the case of TNFα, KD were more sensitive to FU treatment than were WT and Emp (Supplemental Fig. S5 and Fig. 5C). We also confirmed this by measurement of the free DNA level, an indicator of apoptosis, by ELISA (data not shown). These results suggest that constitutive NF-κB activation was involved in resistance to chemotherapy, such as with FU.

Next, LOVO-WT, Emp, and KD were implanted s.c. into nude mice, and when the tumor diameter reached 5 mm, 100 mg/kg of FU was administered i.p. The toxicity of the therapy, such as diarrhea, or therapy-related death was not observed. Obvious body weight loss was not observed in each group. After 35 days, the volumes of the LOVO-WT tumors with FU were about 50% of those without FU. FU administration in KD-implanted mice was very effective. KD tumors with FU showed almost no growth during the observation period, and the volume was about 23% of the LOVO-KD without FU and about 6% of the LOVO-WT tumors without FU (Fig. 5D). Tumor growth of WT and Emp was almost same. Levels of IL-8 in xenograft tumors and serum of tumor-bearing mice were measured. IL-8 levels were higher in WT tumors and serum of WT-implanted mice than in KD tumors and serum of KD-implanted mice (Supplemental Fig. S6). DLD-1-WT and KD were also

![Fig. 4. Angiogenic effects of tumor cells with constitutively active NF-κB.](Image)

- Fig. 4. Angiogenic effects of tumor cells with constitutively active NF-κB.
  - A. Angiogenesis was evaluated by incubating HUVECs in the supernatants of WT and KD (×400). HUVECs were incubated for 72 h in the supernatants of DLD-1-WT, DLD-1-Emp, DLD-1-KD, LOVO-WT, LOVO-Emp, and LOVO-KD.
  - B. C. IL-8 and/or MCP-1 were knocked down by siRNA in DLD-1 and LOVO, and HUVECs were incubated in the supernatant. To evaluate angiogenesis, the number of branch points was counted. Results are averages ± S.E. Asterisks, significant difference observed by two-sided ANOVA with Dunnett’s multiple comparison.
  - D. Semiclosed silicone cylinders (angioreactors) with the supernatants of WT and KD were implanted s.c. into mice for 14 d. Tubes were harvested and the numbers of blood vessels were counted. Results are averages ± S.E. Asterisks, *P < 0.05 by Student’s t-test.
implanted s.c. into nude mice, and when the tumor diameter reached 5 mm, 100 mg/kg of FU was administered i.p. The growth curves of DLD-1 cells were similar to that of LOVO cells (Supplemental Fig. S7).

**Discussion**

It has been reported that constitutive activation of NF-κB is frequently observed in tumors, including leukemias and...
lymphomas, as well as solid tumors, such as prostate, colorectal, and pancreatic carcinomas (8, 19, 20). This activation in tumor cells leads to overexpression of a variety of NF-κB target genes, conferring growth advantages, such as antiapoptosis- and cell cycle–promoting genes. In addition, constitutive activation of NF-κB is associated with strong resistance to chemotherapy and radiotherapy (21).

We showed that secretion of angiogenic factors and antiapoptotic effects may be important mechanisms of tumor-mediated constitutive NF-κB activity. VEGF is believed to be one of the most important factors in tumor angiogenesis (22). It has been reported that VEGF is constitutively secreted from certain tumor types and that its expression can be induced under hypoxic conditions dependent on hypoxia-inducible factor (23). Anti-VEGF therapy for colorectal cancer has been used clinically, but it does not show long-term effects (24, 25). In advanced colon cancer, tumor angiogenesis is thought to be the most important factor for tumor growth. In addition to VEGF, many other factors involved in angiogenesis have been reported (22). However, the effects of these factors on angiogenesis remain unknown in vivo.

Here, we propose that NF-κB inhibition may represent a potent antiangiogenic treatment modality in colorectal cancer, especially in cases with constitutive NF-κB activation. Microarray and protein array analyses showed that the levels of several chemokines associated with angiogenesis were increased in cells with constitutive NF-κB activation (26–28). However, there have been few studies examining the significance of such chemokine secretion in clinical cases. Using surgically resected specimens of colorectal carcinomas and adjacent normal tissues, IL-8 and MCP-1 levels were shown to be significantly increased in >30% of tumor specimens. In addition, specimens with high IL-8 levels and with high MCP-1 levels tended to coincide, as seen in colorectal carcinoma cell lines. Although we have not yet analyzed other candidates, such as CXCL1 or CXCL2 (29), it seems that there are no unique factors among NF-κB-regulated genes involved in angiogenesis. However, inhibition of NF-κB, a master regulator of these candidates, is sufficient to inhibit angiogenesis mediated by tumor cells. In this regard, NF-κB inhibition may be a potent form of antiangiogenic therapy for colorectal cancer.

Second, NF-κB activation is also a master regulatory step in antiapoptosis. Several mechanisms have been reported regarding this antiapoptotic effect of NF-κB activation (30). NF-κB exerts its prosurvival activity primarily through the induction of target genes, the products of which inhibit components of the apoptotic machinery. These include FLIP, Bcl-XL, cellular inhibitor of apoptosis (c-IAP), and X chromosome-linked inhibitor of apoptosis (XIAP; refs. 30, 31). FLIP inhibits apoptosis by interfering with caspase-8 activation. c-IAP and XIAP bind directly to and inhibit effector caspases. In addition, decreased NF-κB activity and elevated JNK activity have been shown to promote cell death through ROS accumulation (15, 16, 32). In this study, we showed that constitutive activation of NF-κB promoted antiapoptotic effects against TNFα and FU in tumor cells. Microarray and real-time PCR analyses indicated that the levels of expression of apoptosis-related genes, such as A20 and cIAP2, were decreased by inhibition of constitutive NF-κB activation, whereas apoptosis-related genes, such as cIAP1 and CYLD, known to be regulated by NF-κB activation, were apparently unaffected. It remains to be determined whether gene expression regulated by NF-κB activity differs between inducible and constitutive activation. We also showed that constitutive activation of NF-κB promoted the antiapoptotic function against FU in vivo. This increased sensitivity of FU treatment by inhibition of NF-κB activation has been reported in many cancer cell types, suggesting that the effect is not only in cells with constitutively active NF-κB activity, but also in those with FU-inducible NF-κB activation (33). These results suggest that combination therapy with FU and an inhibitor of NF-κB should be effective in tumors both with and without constitutively active NF-κB.

We have not analyzed the mechanism(s) leading to constitutive activation of NF-κB. The fundamental reason for the constitutive activation of NF-κB in tumors remains unclear. However, in hematopoietic tumor cells, such as BCL10 and MALT1, NF-κB is activated and has been implicated in the pathogenesis of mucosa-associated lymphoid tissue lymphomas (34). In multiple myeloma, a majority of myeloma samples and cell lines had elevated NF-κB target gene expression, associated with genetic or epigenetic alterations in NIK, TRAF3, CYLD, CD40, NFKB1, or NFKB2 (35, 36). CYLD was originally identified as a tumor suppressor that is mutated in familial cylindromatosis and plays a role in NF-κB regulation. Hellerbrand et al. reported that CYLD was downregulated or lost in all tumor cell lines investigated as compared with primary human colonic epithelial cells (37). In addition, Zhang et al. reported that CYLD-deficient mice were more susceptible to induction of colonic inflammation and showed a marked increase in the incidence of tumors as compared with controls in a colitis-associated cancer model (38). We analyzed CYLD mRNA and protein expression, but found no difference between WT and KD (data not shown). In addition, it has been reported that basal levels of NF-κB activity were not different between wild-type and CYLD-deficient cells.

The application of an inhibitor of NF-κB may prove useful as anticancer therapy. Given the antiapoptotic effect of NF-κB, its inhibition should result in apoptosis. Furthermore, constitutive NF-κB activation is involved in tumor angiogenesis, as suggested by this study. Currently, a great deal of research effort is being devoted to the development of IKKβ/NF-κB inhibitors (39). Recently, to test NF-κB inhibitors for potential clinical applicability, we showed that the NEMO-binding domain peptide, an amino-terminal α-helical region of NEMO, associated with a carboxyl-terminal segment of IKKα and IKKβ (40) and has been shown to block the association of NEMO with IKKβ and to inhibit NF-κB activity and reduced inflammatory injury (41) and the incidence of colitis-associated cancer (data not shown) in mice. These results indicate that IKKβ-targeted NF-κB blockade, using the NEMO-binding domain peptide, may be a reasonable therapeutic approach for inflammatory bowel disease and colitis-associated cancer. In addition to the NEMO-binding domain peptide, as described above, several NF-κB inhibitors, such as proteasome inhibitors, BAY11, the soy isoflavone genistein, parthenolide, and dehydroxymethylepoxyquinomicin, have been developed. It has also been shown that steroids and nonsteroidal anti-inflammatory drugs block NF-κB (42). However, few cell types will undergo apoptosis solely via NF-κB inhibition, and it is likely that a NF-κB inhibitor will be effective only in combination with other anticancer agents or radiotherapy. As activation of NF-κB by some agents or stimuli may mediate apoptosis under
certain conditions, it will be necessary to investigate the function of NF-κB, proapoptotic or antiapoptotic, before the use of inhibitors against such tumors.

In summary, constitutive activation of NF-κB is frequently observed in colon cancer, and plays a key role in angiogenesis and antiapoptosis, promoting tumor growth. Molecular targeted therapy against NF-κB activation should be effective in colorectal carcinomas, especially those with constitutive NF-κB activation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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Kei Sakamoto, Shin Maeda, Yohko Hikiba, et al.


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