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Increased Nuclear Factor-kB Activation in Human Colorectal Carcinoma and its Correlation with Tumor Progression

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Abstract. *Objectives: Nuclear factor-kB (NF-kB) is a transcription factor that participates in the induction of several genes for cytokines and enzymes that play important functional roles in various cell types. The aim of this study was to determine NF-kB activation status in human colorectal carcinoma and its correlation to the clinicopathological characteristics of patients. Materials and Methods: We examined the activation status of NF-kB in 28 resected colorectal carcinomas and in colonic mucosa from uninvolved portions of these specimens by electrophoretic mobility shift assay (EMSA) and immunohistochemical staining for the p65 subunit of NF-kB. Results: EMSA showed much greater activation in the tumors than in normal mucosa, as did epithelial p65 immunostaining. NF-kB activation significantly increased in the more progressed cases (T3+T4 cases or Stage II< cases). In vitro studies using lipopolysaccharide (LPS)-responsive colon carcinoma cells suggested a correlation between NF-kB activation and cell proliferation. Conclusion: Our findings indicated that NF-kB is constitutively activated in human colorectal carcinoma tissue and correlates with tumor progression. The regulation of this transcription factor might be therapeutically useful against these tumors.*

Recent studies suggest that nuclear factor-kB (NF-kB), a transcription factor of the Rel/NF-kB family, may be an important determinant of the biological characteristics of tumor cells (1). NF-kB was first identified as a regulator of kappa light-chain gene expression in murine B lymphocytes (2). The activated form of NF-kB is a heterodimer that usually consists of two proteins, a p65 subunit and a p50 subunit. In resting cells, NF-kB is located in the cytoplasm where it is bound to IkB, which prevents it from entering the nucleus. When the cell is stimulated, IkB is phosphorylated by IkB kinase, which is followed by proteolytic removal of IkB. NF-kB, now activated, is translocated to the nucleus where it acts as a positive regulator of target genes (3). NF-kB has been demonstrated to induce genes for many cytokines, enzymes and adhesion molecules (4). Activation of NF-kB has been shown to be a critical step in cyclooxygenase-2 (COX-2) activation in macrophages exposed to lipopolysaccharide (LPS) and also in inducible nitric oxide synthase (iNOS) expression (5,6).

Prostaglandin E_2 (PGE_2), a product of COX-2, is overproduced in some cancer patients, in whom it mediates immunosuppression (7). NO produced by NOS has also been implicated in tumor-related effects, such as angiogenesis, regulation of microcirculation, cellular injury and immunosuppression (8,9). Both COX-2 and iNOS have recently been shown to be overexpressed in colon carcinoma tissues (10-12); the mechanisms underlying this overexpression remain unclear, particularly whether NF-kB could be involved.

Recently, we have reported for the first time that LPS increases COX-2 expression in a certain colon carcinoma cell line through NF-kB (13). Our hypothesis is that NF-kB is continuously activated in colorectal carcinoma tissue samples, which have ongoing exposure to enteric bacteria and their toxic product, LPS, as well as inflammatory cytokines that can induce NF-kB activation. In the present study, we demonstrated that NF-kB is constitutively activated in colorectal carcinoma tissues using an electrophoretic mobility shift assay (EMSA) and immunohistochemical staining. We also reported that NF-kB activation correlates with cancer progression .

Materials and Methods

Patients and tissue samples. Specimens from 28 patients with colon cancer were studied after the patients' informed consent was obtained (Table I). Both carcinoma and nonneoplastic tissue samples were obtained from respective areas in colonic resection specimens. Tissue samples were stored at -80˚C for nuclear extraction and EMSA, or

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Table I. *Clinicopathological features of colorectal carcinoma patients.*

Parameter	Number of cases
Total cases	28
Male / female	17 / 11
Mean age (range)	64 (34-90)
Tumor site (A / T / D / S / R)	3 / 2 / 5 / 8 / 10
Degree of differentiation (well / moderately / poorly)	17 / 9 / 2
Depth of invasion* (T1 / T2 / T3 / T4)	3 / 6 / 11 / 8
Lymph node metastasis* (N0 / N1 / N2)	10 / 7 / 11
Distant metastasis* (M0 / M1)	22 / 6
Stage* (I / II / III / IV)	4 / 6 / 12 / 6

A = ascending colon including cecum; T = transverse colon; D = descending colon; S = sigmoid colon; R = rectum.
* Each factor was determined according to the TNM classification (14).

were fixed by immersion in neutral buffered formalin (Mildform; Wako Pure Chemical Industries, Osaka, Japan) for both histological diagnosis and immunohistochemistry.

Cell lines. The human colon adenocarcinoma CE-1 cell line was established in our laboratory from a surgical specimen (moderately-differentiated adenocarcinoma of the descending colon, T4T2M0 Stage III according to the TNM classification) (13,14). A human colon carcinoma cell line, WiDr, was obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were maintained in RPMI-1640 medium (Sanko Pure Chemicals, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Filtron Pty. Ltd., Australia) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37˚C.

Nuclear extract preparation. All steps for nuclear extraction were performed at 4˚C using prechilled buffers with protease inhibitors as follows: 10 mM benzamidine, 0.7 mg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 2 μg/ml antipain, 0.7 μg/ml pepstatin, 0.5 mM PMSF and 0.5 mM AEBSF (all purchased from Sigma Chemical, St. Louis, MO, USA) as previously described (15). Colorectal tissue samples or tumor cells were homogenized in buffer A containing 300 mM sucrose, 60 mM KCl, 15 mM HEPES (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine and 14 mM β-mercaptoethanol using a motorized homogenizer. Homogenates were centrifuged at 800 xg for 5 min. Pellets were resuspended in buffer A containing 0.5% NP-40, and these resultant suspensions were layered over buffer B (same as buffer A except containing 30% sucrose) and then centrifuged for 5 min at 1600 xg to pellet the nuclear protein. Based on Gorski's preparation procedure (16), samples finally were added to buffer D containing 20 mM HEPES (pH 7.5), 0.2 mM EDTA, 0.1 M KCl and 20% glycerol, and then centrifuged at 4˚C to remove insoluble materials. Extracts were frozen at -80˚C until assay. Protein concentrations were determined by the method of Bradford using a kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

EMSA analysis. Nuclear protein extracts of the carcinoma and adjacent nonneoplastic tissues were analyzed using an EMSA to determine NF-kB nuclear translocation as reported previously (15). Nuclear protein (10 μg in each assay) was incubated at room temperature for 30 min with 0.2 μg of ^{32}P-end-labeled double-stranded oligonucleotide containing the NF-kB binding motif (Promega, Madison, WI, USA) and 1 μg of poly (dI-dC) as an inhibitor of nonspecific binding, in binding buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid (HEPES; pH 7.4), 60 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmehylsulfonyl fluoride, 1% Nonidet P-40 and 8% glycerol. The sequence of the double-stranded oligomers used for EMSA was 5'-AGT TGA GGG GAC TTT CCC AGG C-3'. In a cold-competition experiment, unlabeled oligonucleotide was incubated with extracts for 30 min at room temperature prior to the addition of the radiolabeled probe. The reaction mixtures were electrophoresed through 5% polyacrylamide Tris-glycine-EDTA gels. After the gels were dried, DNA-protein complexes were visualized by autoradiography.

Immunohistochemistry. Tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at a thickness of 4 to 5 μm and then deparaffinized. Slides were immersed in 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity and then in normal goat serum (1.5%) for 20 min to block nonspecific binding sites. Immunostaining was performed at 4˚C overnight with a rabbit polyclonal IgG specific for the p65 subunit of NF-kB (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:150. Sections were treated with biotinylated secondary antibodies at a dilution of 1:200 (Nichirei Co., Ltd., Tokyo, Japan) and antibody-binding sites were visualized by an avidin-biotin-peroxidase complex solution and 3,3'-diaminobenzidine (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Nonimmune rabbit serum was used as a negative control.

Cell proliferation assay. To analyze the growth of cells, a standard [^{3}H]thymidine incorporation assay was set up in 96-well round-bottomed microplates as described previously (17). Tumor cells were seeded in wells at 2 x 10^4 cells/well in 200 μl of culture media with or without LPS (100 ng/ml), incubated for 68 h, after which [^{3}H]thymidine was added to each well (0.5 mCi/well). The plate was incubated for an additional 12 h. Cells were harvested with a cell harvester (LKB-Wallac, Turku, Finland) and radioactivity was measured in a scintillation counter (LKB-Wallac). Analysis of cell proliferation was carried out in triplicate.

Statistical analysis. Results are expressed as the mean ± standard error. Differences were assessed statistically using Student's *t*-test with $p<0.05$ considered significant.

Results

NF-kB activation in tumor tissues of colorectal carcinomas. NF-kB activation in colorectal carcinoma tissues was compared with that in adjacent nonneoplastic tissues using EMSA. Figure 1A shows EMSA results for 5 representative cases. Although NF-kB was slightly activated in nontumor tissues, nuclear translocation of NF-kB was distinctly greater in all carcinomas than in nonneoplastic tissue from the same cases. To confirm specificity of binding, competitive studies were performed using a 100-fold excess of unlabeled oligonucleotide; this

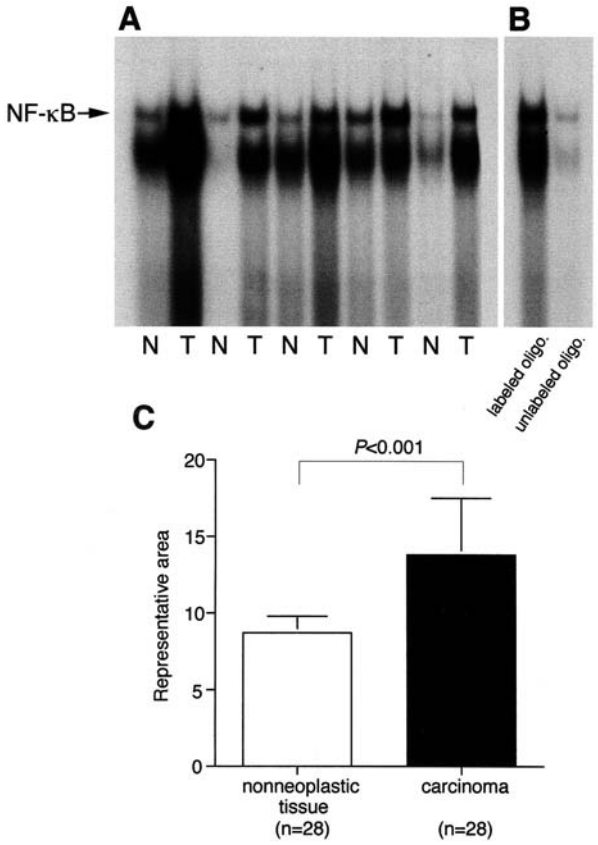

Figure 1. *NF-kB activation in colon carcinoma specimens. (A) EMSA analysis in five representative cases comparing NF-kB in nuclear proteins from carcinomas and nonneoplastic tissues (lane N, normal tissue; lane T, tumor tissue). (B) Competition experiments performed with excesses of unlabeled oligonucleotide (oligo.). (C) Quantitative autoradiographic data for NF-kB activation in carcinomas and nonneoplastic tissues. Data are the mean ± standard error.*

resulted in a diminished band intensity, indicating that binding of the NF-kB probe was specific (Figure 1B). Densitometric analysis of autoradiographs demonstrated that NF-kB nuclear translocation was significantly greater in carcinomas than in nonneoplastic tissue (n=28, 13.79 ± 3.69 *vs.* 8.69±1.14, $p<0.001$; Figure 1C).

Thus, activation of NF-kB in colorectal carcinomas was significantly increased beyond activation in nonneoplastic tissues.

Correlation between NF-kB activation and clinicopathological factors of patients. The NF-kB activation levels in tumor tissues, as subdivided according to the TNM classification and cancer stage, are demonstrated in Figure 2. NF-kB activation levels were significantly higher in the T3+T4 subgroup as compared to that in the T1+T2 subgroup (15.95±2.17 *vs.* 9.22±0.83, $p<0.001$; Figure 2A). NF-kB activation significantly increased in the Stage II< subgroups as compared with that in the Stage I subgroup (Stage I *vs.* Stage II, 8.75±0.96 *vs.* 14.67±1.63, $p<0.001$; Stage I *vs.* Stage III<, 8.75±0.96 *vs.* 14.79±3.68, $p=0.004$; Figure 2D). No significant correlations were detected between NF-kB activation and the presence of lymph node metastasis or distant metastasis (Figure 2B,C). Thus, significantly increased NF-kB activation could be observed in the more progressed subgroups, such as the T3+T4 subgroup and Stage II< subgroup.

NF-kB (p65 protein) expression by immunohistochemistry. Activation of NF-kB in colorectal carcinoma was also determined at the protein level, using a polyclonal antibody against the NF-kB subunit p65 in samples where NF-kB was strongly activated according to EMSA. Strong anti-p65 immunoreactivity was detected in the nuclei of most carcinoma foci (Figure 3C, D), while in nonneoplastic tissues immunoreactivity for p65 was weak in stromal cells but nearly absent in normal epithelial cells (Figure 3A, B). Immunoreactivity was seen throughout the tumor sections, which ranged from well- to poorly-differentiated adenocarcinoma. These observations demonstrate NF-kB activation in colorectal carcinoma cells at the protein level.

Activated NF-kB effects on colon carcinoma cell proliferation. To confirm the effect of activated NF-kB on tumor progression, we examined colon carcinoma cell proliferation using CE-1 cells of which NF-kB was activated by LPS stimulation (13). LPS-unresponsive WiDr cells were used as control. EMSA revealed that NF-kB was constitutively activated and LPS up-regulated activation of NF-kB in CE-1 cells (Figure 4A). Although NF-kB also was activated in WiDr cells, LPS did not affect nuclear translocation of NF-kB. In LPS-responsive CE-1 cells, [^{3}H]thymidine uptake significantly increased with the up-regulation of NF-kB activation by LPS (Figure 4B). WiDr cells showed no change of [^{3}H]thymidine incorporation by LPS stimulation. These *in vitro* studies suggest a correlation between NF-kB activation and colon carcinoma cell proliferation *via* DNA synthesis.

Discussion

In this study we demonstrated, by EMSA and immunohistochemistry, that human colorectal carcinoma tissues show a greater level of nuclear translocation of NF-kB, than do nonneoplastic tissues. These data suggest that NF-kB is constitutively activated in human colon carcinomas. The NF-kB activation level correlates with the degree of carcinoma progression.

The mechanisms by which NF-kB is activated in colon carcinoma have not been fully described. NF-kB activation

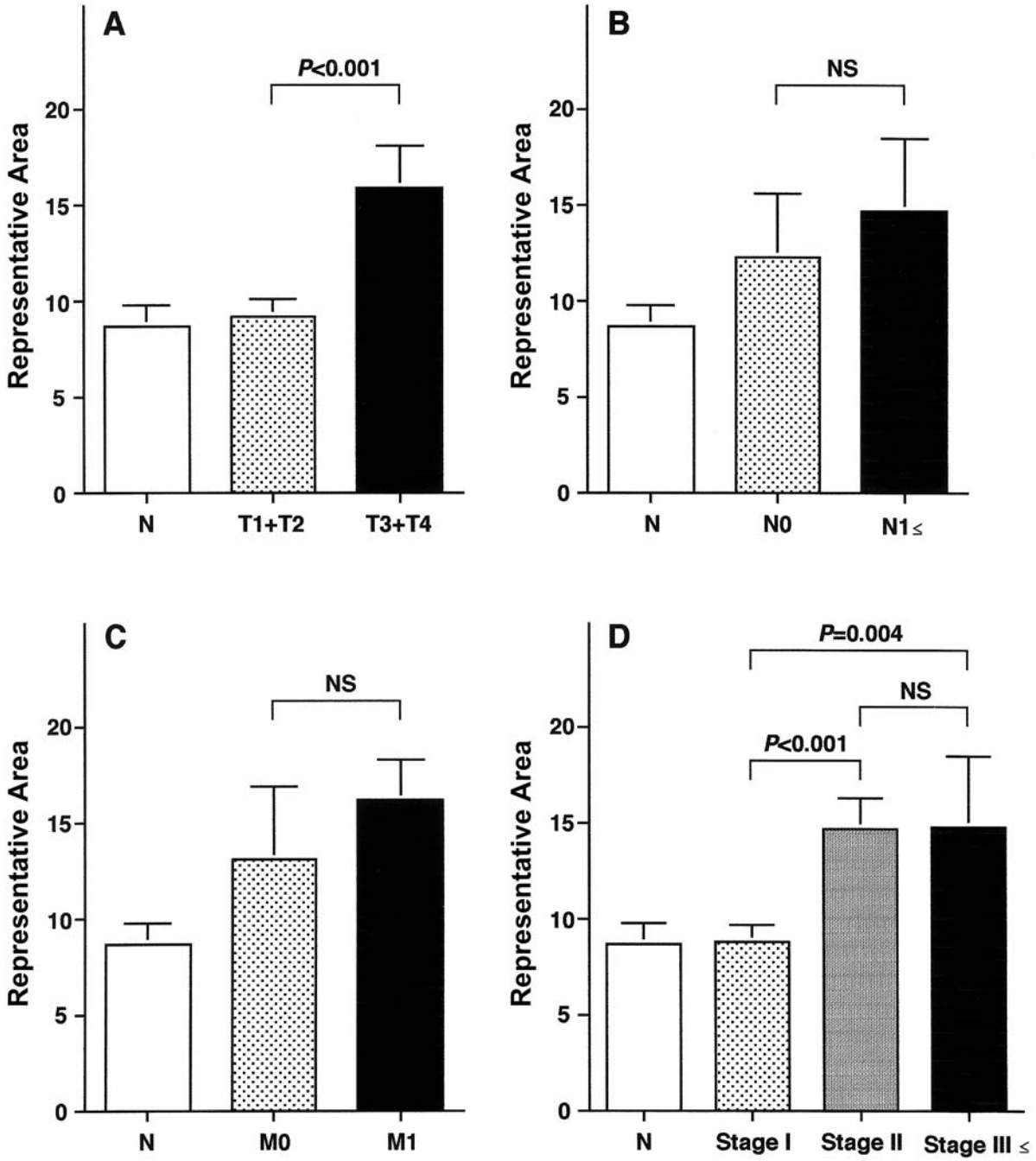

Figure 2. *Quantitative autoradiographic data for NF-kB activation of (A) the T1+T2 and the T3+T4 subgroups, (B) the N0 and the N1< subgroups, (C) the M0 and the M1 subgroups, and (D) the Stage I, the Stage II and the Stage III< subgroups as well as that of the normal tissue group (N). Data are the mean ± standard error. NS, not significant. Each factor was determined according to the TNM classification (14).*

is a complex process that can be triggered by many agents, including inflammatory cytokines, mitogens, bacterial products, protein synthesis inhibitors, reactive oxygen species, ultraviolet light and phorbol esters (4,18). One of these stimuli is LPS, which is released from the surfaces of cell membranes of Gram-negative bacteria. LPS triggers potent inflammatory response, including induction of inflammatory cytokines. Very recently, we found for the first time that LPS can induce activation of NF-kB in a certain colon carcinoma cell line (13). Since the colon contains

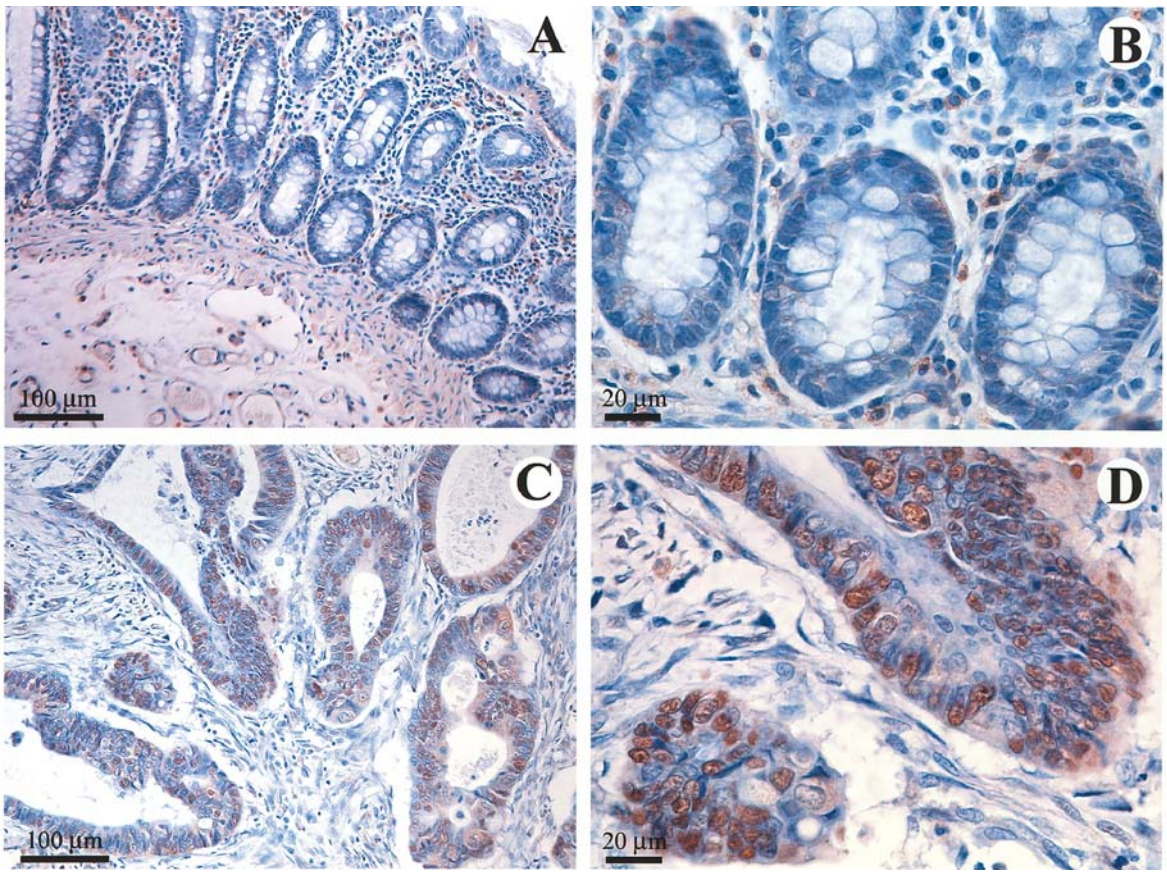

Figure 3. *Immunohistochemical staining for the NF-kB subunit p65 in colon carcinoma. (A, B) Nonneoplastic tissue. While weak immunoreactivity for p65 was detected in stromal cells including lymphocytes, reactivity was essentially absent in normal epithelial cells. (C, D) Carcinoma tissue. Carcinoma foci showed strong anti-p65 immunoreactivity in nuclei.*

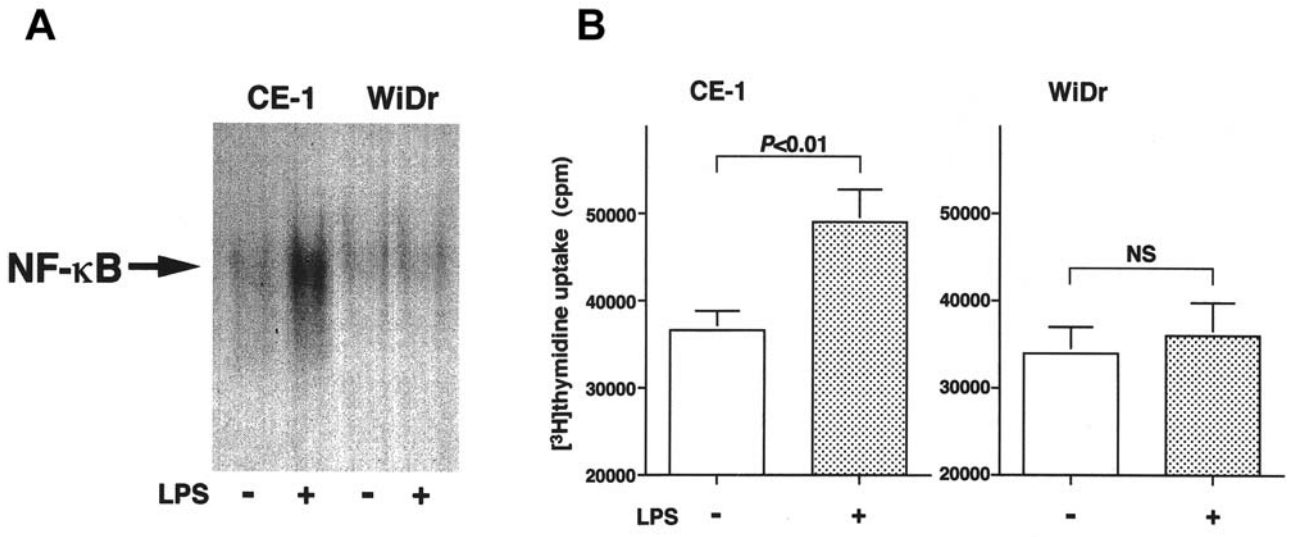

Figure 4. *NF-kB activation and proliferation of colon carcinoma cell lines by LPS stimulation. (A) EMSA study and (B) cell proliferation using [^{3}H]thymidine uptake of CE-1 and WiDr cells with or without LPS treatment. Data are the mean ± standard error. NS, not significant.*

large numbers of Gram-negative bacteria, colonic epithelial cells would be heavily exposed to LPS, as would the cells of carcinomas developing in the colon. Therefore, local production of LPS and inflammatory cytokines could result in increased activation of NF-kB in colorectal carcinoma.

In chronic inflammatory disease NF-kB is known to regulate many genes for cytokines, enzymes and adhesion molecules (4). However, the role of NF-kB in tumor development remains unresolved. Activation of NF-kB in tumor cells has been linked to malignant potential (19). In our study, NF-kB activation significantly increased in clinically progressed carcinoma samples. NF-kB activation also was correlated with tumor cell proliferation *in vitro*. Giri *et al.* (20) recently have demonstrated that NF-kB activation in HuT-78 T cell lymphoma cells was associated with reduction in apoptosis. Nakshatri *et al.* (21) showed that breast cancers that lack the estrogen receptor overexpress NF-kB-regulated genes and also found that NF-kB protects breast carcinoma cells against induction of apoptosis by tumor necrosis factor-α, ionizing radiation and the chemotherapeutic agent daunorubicin. NF-kB may also regulate iNOS and COX-2, which have been found to be increased in colorectal carcinoma (10-12); both iNOS (8,9) and COX-2 (22,23) recently have been implicated in such tumor-related processes as carcinogenesis, angiogenesis, metastasis and immunosuppression. In this context, our results suggest that overexpression of iNOS and COX-2, which can enhance malignant potential in colorectal carcinoma, may result from NF-kB activation.

NF-kB has been shown to be constitutively activated in pancreatic adenocarcinoma or hepatocelluler carcinoma tissues (24,25). If NF-kB is an important mediator of progression in carcinoma, regulation of this factor may be useful in treating the tumor. Some antioxidants or protease inhibitors have been investigated for effects against NF-kB (26,27). Since NF-kB is critical to the immune response and other host defenses, global inhibition over prolonged periods may be undesirable. However, NF-kB often works together with other transcription factors. If tumor-specific inhibition could involve a limited range of cell types or certain synergistic interactions with other transcription factors, targeting of NF-kB might become a practical way to restrict the malignant potential of carcinoma.

In conclusion, we demonstrated for the first time constitutive NF-kB activation in human colorectal carcinomas using clinical samples and its correlation with tumor progression. NF-kB activity may play a key role in colorectal carcinoma progression through activation of its downstream target genes. Further investigation into the mechanism of NF-kB activation may help to provide new therapeutic potential for colorectal cancer patients.

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