Nuclear Factor-κB p65 (RelA) Transcription Factor Is Constitutively Activated in Human Gastric Carcinoma Tissue

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ABSTRACT

Purpose: Activation of transcription factor nuclear factor-κB (NF-κB) has been shown to play a role in cell proliferation, apoptosis, cytokine production, and oncogenesis. The purpose of this study was to determine whether NF-κB is constitutively activated in human gastric carcinoma tissues and, if so, to determine any correlation between NF-κB activity and clinicopathological features of gastric carcinoma.

Experimental Design: NF-κB activation was determined by immunohistochemical analysis of formalin-fixed, paraffin-embedded specimens from 64 gastric carcinoma patients. We quantified nuclear staining of RelA as a marker of NF-κB activation.

Results: Nuclear translocation of RelA was significantly high in tumor cells in comparison to that in adjacent normal epithelial cells (22.5 ± 2.4% versus 8.6 ± 1.5%, P < 0.0001). There was a significant correlation between NF-κB activation (nuclear translocation of RelA) and expression of urokinase-type plasminogen activator, an invasion-related factor and target of NF-κB in tumor cells (ρ = 0.393; P = 0.0013). NF-κB activation was correlated with tumor invasion-related clinicopathological features such as lymphatic invasion of tumor cells (P = 0.0126), depth of invasion (P = 0.0539), peritoneal metastases (P = 0.0538), and tumor size (P = 0.0164).

Conclusions: Collectively, the data show that NF-κB is constitutively activated in human gastric carcinoma tissues and suggest that NF-κB activity is related to tumor progression due to its transcriptional regulation of invasion-related factors such as urokinase-type plasminogen activator.

INTRODUCTION

Rel/NF-κB is a family of dimeric transcription factors that control the expression of numerous genes involved in cell growth, differentiation, regulation of apoptosis, cytokine production, and neoplastic transformation (1, 2). The Rel/NF-κB family comprises NF-κB1 (p50), NF-κB2 (p52), and the Rel proteins, RelA (p65), RelB, and c-Rel, which have a high level of sequence homology within their NH2-terminal 300 amino acids, the Rel homology domain (3). p50 and p52 can interact with the RelA proteins to form all possible homo- and heterodimer combinations (2). The most common dimer is the RelA (p65)/NF-κB1 (p50) heterodimer, i.e., NF-κB. In most unstimulated cells, Rel/NF-κB proteins are sequestered in the cytoplasm and are complexed with specific inhibitor proteins called IκBs that render the Rel/NF-κB proteins inactive (2, 3). Stimulation of cells leads to phosphorylation and degradation of IκBs and allows translocation of Rel/NF-κB to the nucleus, resulting in expression of target genes (4). A surprising variety of inducers have been found to activate Rel/NF-κB (3). These pathways are involved in innate immune responses that involve cytokines such as tumor necrosis factor (TNF)-α and IL-1 (5), responses to physical stresses such as UV light and ionizing radiation (X and γ), and responses to oxidative stresses such as hydrogen peroxide and butyl peroxide (6, 7).

Several investigators have reported constitutive activation of NF-κB in various types of human tumor cell lines, including those of lymphoid origin such as Hodgkin/Reed Sternberg cells (8), T-cell lymphoma Hut 78 cells (9), and multiple myeloma cells (10). In addition, nonlymphoid cell lines including ovarian cancer cells (11), lung carcinoma cells (12), breast cancer cells (13), thyroid carcinoma cells (14), melanomas (15), and bladder cancer cells (16) exhibit enhanced NF-κB activity.

Recent studies have also indicated that NF-κB is constitutively activated in tumors such as pancreatic cancer and breast cancer (17, 18). However, little information is available concerning NF-κB activation in gastric carcinoma, which is one of the most aggressive forms of cancer. Several invasion-related factors, including uPA, are overexpressed in gastric carcinoma
Overexpression of uPA in cancer cells is thought to contribute to tumor growth, invasion, and metastasis. However, the mechanism by which uPA is overexpressed in cancer cells remains unclear. It was reported recently that uPA expression is regulated by NF-κB in several tumor cell lines (20, 21). Although these findings suggest that continuous activation of NF-κB in tumors may contribute to aggressive characteristic features of tumor aggression via transcription of invasion-related factors, there is no definitive data showing a correlation between NF-κB activation and clinicopathological features of tumors. We undertook the present study to determine whether NF-κB is constitutively activated in gastric carcinoma tissues, to examine whether uPA expression is regulated by NF-κB activation, and to evaluate the correlation between NF-κB activity and clinicopathological features as well as survival in gastric carcinoma.

MATERIALS AND METHODS

Clinical Samples. Sixty-four gastric carcinoma patients who gave informed consent before surgical treatment were entered into the present study. Both tumor and adjacent normal tissue specimens were obtained from surgically resected tissues. Tissues were fixed with 10% formalin and embedded in paraffin for simple H&E staining and immunohistochemistry. In several cases, tumor and nontumor samples were collected in the operating room, and nuclear proteins were isolated immediately. All specimens were classified histologically according to the General Rules for the Gastric Cancer Study of the Japanese Society for Gastric Cancer (22).

Cell Culture. Human gastric cancer cell line HTB-135 was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 (Sanko Pure Chemicals, Tokyo, Japan) supplemented with 10% fetal bovine serum (Filtron Pty. Ltd., Brooklyn, Victoria, Australia) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in an atmosphere of 5% CO₂ in air.

Immunohistochemistry. Immunostaining was performed as described previously but with a slight modification (23). Briefly, slides were probed with either anti-RelA (p65; 1:150; sc-109; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-uPA (1:100; sc-6830; Santa Cruz Biotechnology) and incubated with secondary antibodies (RelA, goat antirabbit immunoglobulin; uPA, rabbit antigoat immunoglobulin; Nichirei Co., Ltd., Tokyo, Japan). Finally, antibody binding was detected with a combination of DAB (40 mg/150 ml in PBS; Wako Pure Chemical Industries, Hyogo, Japan) and 0.06% hydrogen peroxide (H₂O₂).

The numbers of cytoplasmic- and nuclear-positive cells were counted separately. One hundred cells were counted for each section. Nuclear staining, which indicated nuclear translocation of RelA, was considered a marker of NF-κB activation.

Table 1  Correlation analysis between NF-κB activation and clinicopathological parameters for 64 gastric carcinoma specimens

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± SE</th>
<th>n</th>
<th>P (one-sided)</th>
</tr>
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<tr>
<td>Age</td>
<td>62.4 ± 1.5</td>
<td>64</td>
<td>0.2131</td>
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<tr>
<td>Tumor size (cm)a</td>
<td>6.7 ± 0.6</td>
<td>18</td>
<td>0.1312</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Surgical treatment</td>
<td></td>
<td>30</td>
<td>0.0328</td>
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<tr>
<td>Curabilityc</td>
<td></td>
<td>48</td>
<td>0.1861</td>
</tr>
<tr>
<td>Tumor locationd</td>
<td></td>
<td>24</td>
<td>0.2526</td>
</tr>
<tr>
<td>Macroscopic typesf</td>
<td></td>
<td>31</td>
<td>0.0739</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
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<td>0.0539</td>
</tr>
<tr>
<td>Cytology</td>
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<td>21</td>
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<tr>
<td>Liver metastases</td>
<td></td>
<td>13</td>
<td>0.0975</td>
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<tr>
<td>Lymphatic invasion</td>
<td></td>
<td>17</td>
<td>0.0126</td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td>47</td>
<td>0.0950</td>
</tr>
<tr>
<td>Pathological stages</td>
<td></td>
<td>44</td>
<td></td>
</tr>
</tbody>
</table>

a Tumor size was measured by using the greatest diameter on the mucosal side.

b Bold values indicate statistical significance.

c Curability A, absolutely; curability B, relatively; curability C, noncurative resection.

d Distal location, lower third; proximal location, upper or middle third.

e Localized, sharply demarcated tumors; invasive, infiltrating tumors into the surrounding wall.

(19).
Immunostaining of Cell Line. HTB-135 cells (1 × 10^5 cells/well) were grown on 8-chamber glass slides. Cells were fixed in 100% methanol for 20 min at −20°C. Slides were then immersed in 3% H₂O₂ in methanol for 30 min. To improve the quality of staining, microwave oven-based antigen retrieval was performed as described previously (24). Cells were incubated with Anti-RelA (1:100; Santa Cruz Biotechnology) for 2 h at room temperature. They were then incubated with the appropriate secondary antibody (Nichirei), and antibody binding was detected with a combination of DAB (Wako Pure Chemical Industries) and 0.06% H₂O₂.

Preparation of Nuclear Extract. Carcinoma and normal samples were collected in the operating room and processed within 30 min. Fresh samples were minced and homogenized in hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.1% NP40, and 5% protease inhibitor (0.2 mM DTT, 10 mM benzamidine, 7 μg/ml leupeptin, 50 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 2 μg/ml antipain, 0.7 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-(2-aminoethyl) benzenesulfonylfluoride)]. HTB-135 cells (1 × 10⁶) were washed once with PBS and resuspended in hypotonic buffer. Homogenized tissues and cell lysates were incubated on ice for 10 min, and extraction of nuclear contents was performed as described previously (25). The protein concentrations of nuclear extracts were determined by Bradford assay (Bio-Rad, Hercules, CA; Ref. 26). The nuclear extracts were stored at −80°C until use.

EMSA. Nuclear protein extracts of carcinomas, adjacent normal tissues, and HTB-135 cells were analyzed by EMSA for NF-κB nuclear translocation as described previously (27). Nuclear protein extracts (10 μg in each assay) were incubated for 30 min at 37°C with binding buffer [60 mM HEPES (pH 7.5), 180 mM KCl, 15 mM MgCl₂, 0.6 mM EDTA, and 24% glycerol], poly(dI-dC) (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and 32P-labeled double-stranded oligonucleotide containing the binding motif of NF-κB (Promega Corp., Madison, WI). The sequence of the double-stranded oligomer used for EMSA was 5’-AGTTGGAGGGGCTTTCCAGGC-3’.

RT-PCR Analysis. Total RNA was extracted from HTB-135 cells by the guanidinium thiocyanate-phenol-chloroform single-step method (28). The primer sequences were 5’-AAGAGTGCACTGGTCATAC-3’ (sense) and 5’-CGAATTGAGGTTCAGGTAC-3’ (antisense) for uPA (29) and 5’-CCACCCA TGCAAAATTTCCATGGCA-3’ (sense) and 5’-CAGTCCAGTCCACC-3’ (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (30). Expected RT-PCR product sizes were 318 bp for uPA and 593 bp for GAPDH. PCR conditions comprised an initial denaturation for 2 min at 94°C followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C. The PCR products were separated on ethidium bromide-stained 1.5% agarose gels.

Statistical Analysis. All statistical analyses were performed with SAS Statistical Software (Release 6.12) on a UNIX workstation. The difference in the mean staining rate between tumor and adjacent normal cells was analyzed by Wilcoxon’s rank-sum test for paired data via the SAS/STAT NPAR1WAY procedure. The correlation between RelA nuclear and uPA cytoplasmic staining rates was analyzed by Spearman’s test via the SAS/STAT CORR procedure. This test was also used to analyze correlation between the RelA nuclear staining rate and the clinical features listed in Table 1. Of the 15 items shown on
Table 1, age and tumor size were treated as continuous variables, and the remaining 13 items were treated as dichotomous variables. Wilcoxon’s test for paired data and all Spearman tests were conducted as one-sided tests. Kaplan-Meier curves were used to estimate survival rates, and survival rates were compared by log-rank test via the SAS/STAT PHREG procedure. In this, the RelA nuclear translocation rate was dichotomized by a cutoff point of 25%.

RESULTS

RelA Staining in Gastric Carcinoma Tissues. To ascertain whether NF-κB is activated in gastric carcinoma tissues, an immunohistochemical analysis of RelA was performed with formalin-fixed, paraffin-embedded gastric carcinoma specimens. RelA staining was significantly enhanced both in the cytoplasm and nuclei of tumor cells in comparison to that in adjacent normal epithelial cells, except in gastric body glands. Normal gastric body glands expressed RelA in the cytoplasm but not in nuclei. A representative tumor specimen showing increased RelA staining in both the cytoplasm and nuclei is shown in Fig. 1. We counted the number of stained cells and calculated the percentage of positively stained cells. Cells containing cytoplasmic and/or nuclear staining were counted separately (Fig. 2A). When all specimens were examined, the percentage of tumor cells and adjacent normal gastric epithelial cells with cytoplasmic staining for RelA ranged from 2–96% (mean ± SE, 70.4 ± 2.6%) and from 0–90% (mean ± SE, 35.1 ± 3.2%), respectively. The mean percentage of tumor cells and normal epithelial cells with nuclear staining of RelA was 22.5 ± 2.4% and 8.6 ± 1.5%, respectively. The cytoplasmic (P < 0.0001) and nuclear (P < 0.0001) staining rates were statistically high in tumor cells in comparison with rates in normal epithelial cells (Fig. 2B).

Increased NF-κB DNA Binding Activity in Gastric Carcinoma Tissues. EMSA was used to confirm the increased nuclear translocation of RelA in gastric carcinoma tissues. Fig. 3 shows increased NF-κB DNA binding activity in tumor tissue compared with that in adjacent normal tissue. To confirm the specificity of NF-κB DNA binding, we performed supershift analysis with antibodies specific for RelA (p65) and NF-κB1 (p50). Arrows to the right of the panel indicate supershifted complexes.

NF-κB Activation and uPA Expression in Gastric Carcinoma Tissues. We examined cytoplasmic expression of uPA in gastric carcinoma specimens by immunohistochemical analysis because expression of the uPA gene is thought to be mediated by NF-κB activation (20, 21, 31). Correlation analysis between NF-κB activation and uPA expression with one-sided Spearman’s test showed a positive relation (r = 0.393; n = 64; P = 0.0013). Representative images showing simultaneous nuclear expression of RelA and cytoplasmic staining of uPA are presented in Fig. 4, A and B.

Correlation between NF-κB Activation and uPA Expression in Gastric Carcinoma Cells. To further investigate the correlation between NF-κB activation and uPA expres-
sion in gastric carcinoma cells, we performed in vitro experiments in the HTB-135 human gastric carcinoma cell line with IL-1β. IL-1β (5 ng/ml) treatment increased nuclear staining of RelA in HTB-135 cells as examined by immunohistochemistry. B, effect of IL-1β (1 or 10 ng/ml) treatment for 2 h on RelA nuclear expression as determined by EMSA. Control, Lane 1; 1 ng/ml IL-1β, Lane 2; 10 ng/ml IL-1β, Lane 3; supershift analysis, Lane 4; competition assay, Lane 5. C, effect of IL-1β (0.1, 1, or 10 ng/ml) for 2 h on uPA mRNA expression as determined by RT-PCR.

**DISCUSSION**

We show here that NF-κB is constitutively activated in gastric carcinoma tissues and that NF-κB activation plays a role in uPA expression in this carcinoma. Our data also suggest that NF-κB activation is correlated with clinicopathological features of tumor aggression (especially the invasive ability) of gastric carcinoma.
Previous studies have shown possible activation of NF-κB in various cultured cell lines and several carcinoma tissues. In most studies, NF-κB activation was evaluated on the basis of nuclear translocation of RelA (p65) and/or NF-κB-1 (p50) as demonstrated by EMSA and/or immunohistochemical analysis. For example, Wang et al. (17) showed constitutive activation of NF-κB in human pancreatic adenocarcinomas by EMSA and immunohistochemistry with a monoclonal antibody that recognizes an epitope overlapping the nuclear localization signal and IκBα binding site on RelA. Sovak et al. (18) showed aberrant nuclear translocation of NF-κB by EMSA in human breast carcinoma tissues. We primarily used immunohistochemistry to detect NF-κB activation in human gastric carcinoma tissues. We used a polyclonal antibody against human RelA (p65) that binds not only the active form of RelA but also the inactive form, and we quantified the staining of nuclear (active form) and cytoplasmic (inactive form) RelA separately to evaluate NF-κB activation (Fig. 2A). With such analysis, nuclear translocation of RelA can be estimated at the single-cell level in carcinoma tissue.

Others have shown overexpression of uPA protein in gastric carcinoma by immunostaining (32) or ELISA (33) and have reported expression of uPA to be a prognostic marker in patients with gastric carcinoma (34). uPA is also known to be downstream of NF-κB activation in several tumor cells (20, 21). We examined the relation between NF-κB activation and expression of the invasion-related factor uPA in tumor cells by immunohistochemistry because it permitted estimation of NF-κB activation (nuclear translocation) and uPA expression at the single-cell level (Fig. 4). In fact, most specimens showing high NF-κB activation also showed high uPA expression; however, specimens showing high uPA expression did not always show high NF-κB activation. Several factors, including AP-1, PEA3, and NF-κB, may induce uPA promoter activity (35, 36). In addition, it has been reported that uPA expression may be controlled by the combined actions of several transcription factors (37). In the present study, we showed that IL-1β, a known inducer of NF-κB activation, enhanced both RelA nuclear translocation and uPA mRNA expression in HTB-135 cells (Fig. 5, A–C). Moreover, we recently found that NF-κB decoy or pyrrolidine dithiocarbamate, which is a NF-κB inhibitor, represses the IL-1β-induced overexpression of uPA in HTB-135 cells (data not shown).

Although it has been suggested that the NF-κB family proteins are involved in proliferation (6, 38), angiogenesis, invasiveness, apoptosis (3, 39, 40), and metastasis of tumor cells, the biological significance of NF-κB activation in carcinoma tissues remains unclear. We analyzed the relation between NF-κB activation, as estimated by nuclear translocation of RelA, and traditional clinicopathological parameters. NF-κB activation showed correlations with tumor size (P = 0.0164), lymphatic invasion (P = 0.0126), depth of invasion (P = 0.0539), and peritoneal metastasis (P = 0.0538; Table 1). These parameters are associated with tumor growth, invasion, and metastasis. This is the first report of a correlation between NF-κB activation and aggressiveness of gastric carcinomas.

In conclusion, we found that the level of nuclear translocation of NF-κB is higher in gastric carcinoma cells than in normal adjacent epithelial cells and that activation of NF-κB in this carcinoma is related to uPA expression and lymphatic invasion. Our findings suggest that NF-κB contributes to the aggressiveness of gastric carcinoma via increased invasion-related factors such as uPA and that NF-κB may be an important target for the development of future anti-invasion therapy in cases of gastric carcinoma.

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