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5-Fluorouracil Suppression of NF-κB Is Mediated by the Inhibition of IκB Kinase Activity in Human Salivary Gland Cancer Cells

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We have recently shown that 5-Fluorouracil (5-FU) suppresses the transcription factor NF-κB in human salivary gland cancer cells (cl-1) by mediating upregulation of IκB-α expression. However, the precise mechanism involved in this action has not yet been elucidated. IκB kinases (IKK-α and IKK-β) are the key components of the IKK complex that mediates activation of NF-κB in response to external stimuli such as cytokines. In addition, NF-κB-inducing kinase (NIK) and mitogen-activated protein kinase kinase kinase 1 (MEKK-1), both of which are the upstream kinases for the IKKs, interact with and activate the IKKs. Thus, we investigated the molecular mechanisms involved in the suppression of NF-κB by 5-FU. Although 5-FU did not affect the expression levels of IKKs, NIK, or MEKK-1, IKK activity in cl-1 cells was suppressed at both 6 h and 12 h after treatment with 2 μg/ml 5-FU. Moreover, when cells were treated with various concentrations of 5-FU for 12 h, the concentration of 2 μg/ml efficiently inhibited the IKK activity as compared to 1, 5, or 10 μg/ml. The expression of Fas-associated death domain-like interleukin 1-converting enzyme-inhibitory protein (FLIP), which acts as an inhibitor of an initiator caspase (caspase-8), was down-regulated by 5-FU treatment in cl-1 cells. Apoptosis, as evidenced by cleavage of poly(ADP-ribose) polymerase through the action of an executioner caspase (caspase-3), was also clearly observed. Thus, these results suggest that 5-FU induction of apoptosis in cl-1 cells may be mediated by suppression of NF-κB via inhibition of IKK activity.

Key Words: **NF-κB; salivary gland cancer cells; 5-FU; apoptosis; IKKs; NIK; MEKK-1.**

5-Fluorouracil (5-FU) is a potent chemotherapeutic agent and is one of the most promising drugs applied in combination therapy for the treatment of head and neck carcinomas including salivary gland cancers (1, 2). The best understood mechanism for the cytotoxic effect of 5-FU is the inhibition of thymidylate synthase, the final enzyme of the *de novo* pathway that converts 2′-deoxyuridine to thymidylate by reductive methylation (3). However, the precise mechanism of cytotoxicity induced by 5-FU has not been fully elucidated.

The dimeric NF-κB transcription factor, whose subunits belong to the Rel family of DNA-binding proteins (4, 5), plays a critical role in immune and inflammatory responses (6–8). Under nonstimulated conditions, NF-κB is retained in the cytoplasm by an inhibitory protein, IκB-α. The binding of IκB-α to NF-κB masks nuclear localization signals in NF-κB and prevents its translocation to the nucleus. Conversely, upon stimulation by external stimuli, mitogen-activated protein kinase (MAP)/extracellular signal-regulated kinase (ERK) kinase kinase 1 (MEKK-1) and the NF-κB-inducing kinase (NIK) are activated, and then activation of IκB kinases (IKKs) occurs. Thereafter, activated IKK-α and IKK-β directly phosphorylate IκB-α, followed by its degradation through the ubiquitin-proteasome pathway. Degradation of IκB-α leads to the nuclear translocation of NF-κB to stimulate the expression of its target genes (9).

The functional role of NF-κB in cell death is still controversial. However, accumulated evidence indicates that NF-κB protects cells from apoptosis induced by tumor necrosis factor (TNF) and various genotoxic agents, indicating that NF-κB has an anti-apoptotic function (10–13). Although the molecular mechanism involved in this anti-apoptotic function of NF-κB is not yet fully understood, recent studies suggest that anti-apoptotic proteins (TNF receptor-associated factor (TRAF)-1, TRAF-2, cellular inhibitor of apoptosis protein (cIAP)-1, and cIAP-2) transcriptionally regulated by NF-κB function cooperatively to suppress apoptosis

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through the inhibition of caspase-8 activity (14, 15). Therefore, the downregulation of anti-apoptotic proteins through the suppression of NF-κB activity would be a promising strategy for inducing apoptosis in cancer cells.

Recently, we have shown that 5-FU induces apoptosis in human salivary gland cancer cells (cl-1) by suppressing NF-κB activity via augmented expression of IκB-α protein, that the expression of TRAF-2 and cIAP-1 was also inhibited by 5-FU, and that 5-FU enhanced the activity of caspase-8 and caspase-3 in these cells (16). However, the mechanism involved in the prevention of degradation of IκB-α protein by 5-FU has not yet been elucidated. Thus, we report here that 5-FU induces the accumulation of IκB-α protein by inhibiting the IKK activity in cl-1 cells. In addition, we demonstrate that both the inhibition of Fas-associated death domain-like interleukin 1-converting enzyme-inhibitory protein (FLIP), which suppresses caspase-8 activity, and the cleavage of one of the apoptosis-associated proteins, poly(ADP-ribose) polymerase (PARP), were clearly observed in 5-FU treated cl-1 cells.

MATERIALS AND METHODS

Cells and media. The metastatic human salivary gland adenocarcinoma cell clone (cl-1) was previously established in our laboratory (17). This cell clone was grown in MEM supplemented with 3% calf serum and 2 mM L-glutamine. Cells were cultured in the presence of 5% CO_2 in an incubator at 37°C.

Cytosolic extract preparations. Cells were seeded on 100-mm plastic Petri dishes (Falcon Labware, Oxnard, CA) in MEM supplemented with 3% serum. Twenty-four hours after seeding, cells were treated with 5-FU (2 μg/ml) (Sigma Chemical Co., St. Louis, MO) for the indicated periods of time, and cytosolic extracts were then obtained by a previously described method (18). The concentration of 5-FU was based on the pharmacologic concentration of this agent in the serum of patients that is necessary for the induction of its anti-cancer properties (19). Cells were washed twice with ice-cold PBS before being resuspended in 400 μl of ice-cold lysis buffer consisting of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes) (pH 7.9), 10 mM KCl, 0.1 mM ethylenediaminetetraacetate (EDTA), 0.1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid (EGTA), 0.5 mM dithiothreitol (DTT), 0.5 mg/ml benzamidine, and 2 mg/ml aprotinin for 15 min. Nonidet P-40 was added to a final concentration of 0.3%, and the lysates were vortexed before being pelleted in a microfuge. The supernatants of this centrifugation were the designated cytosolic extracts.

Western blot analysis of MEKK-1, NIK, IKK-α, IKK-β, FLIP, and PARP. Cytosolic extracts were subjected to electrophoresis in 15% SDS–polyacrylamide gel electrophoresis, then transferred to a nitrocellulose membrane. The membranes were incubated with an anti-MEKK-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), with an anti-NIK antibody (Santa Cruz), with an anti-IKK-α antibody (Santa Cruz), with an anti-IKK-β antibody (Santa Cruz), with an anti-FLIP antibody (Santa Cruz), or with an anti-PARP antibody (Santa Cruz). After intervening rinses with PBS, the antibody was detected using a chemiluminescence Western blotting kit (Amersham, Tokyo, Japan) according to the manufacturer's instructions.

In vitro kinase assay. Cells were untreated or treated with 5-FU (2 μg/ml) for various periods of time, and then washed with ice-cold 5 mM EDTA in PBS, followed by lysing on ice in TN buffer (containing 50 mM Tris (pH 7.5), 250 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 50 mM NaF, 20 mM β-glycophosphate (Sigma), 20 mM *p*-nitrophenyl phosphate (Sigma), 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM EDTA, and 1 mM EGTA). Cells were spun at 4°C 12,000 rpm for 30 min, and cell lysates were immunoprecipitated using an anti-IKK-β antibody (Santa Cruz). Aliquots of the immunoprecipitate were then incubated with 0.5 μg of GST-IκB-α (1–317) (Santa Cruz) protein in 15 μl of kinase buffer (containing 20 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM EDTA, 20 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 20 mM creatine phosphate, and 10 μCi of [γ-^{32}P]ATP) at 30°C for 30 min. Proteins were separated by means of SDS–polyacrylamide gel electrophoresis, and phosphorylated IκB-α proteins were visualized by means of autoradiography.

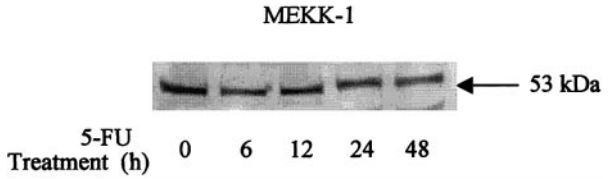

FIG. 1. Western blot analysis for the expression of MEKK-1 protein in cl-1 cells. Cytoplasmic extracts were prepared from cells following 5-FU (2 μg/ml) treatment for the indicated time points. 5-FU treatment did not affect the expression level of MEKK-1 protein in cl-1 cells. The expression level of NIK protein was also not affected by 5-FU treatment (data not shown).

RESULTS

Effect of 5-FU on the Expression of MEKK-1 and NIK Proteins

Since we previously showed that 5-FU prevents the degradation of IκB-α protein in cl-1 cells (16), in the present study we first examined the effect of 5-FU on the expression levels of MEKK-1 and NIK proteins, both of which are the most upstream kinases in the signal transduction of IκB-α phosphorylation (9). As shown in Fig. 1, expression levels of neither protein were affected by 5-FU treatment for up to 48 h (NIK, data not shown).

5-FU Suppression of IKK Activity in cl-1 Cells

Next, we examined the expression of downstream kinases of MEKK-1 and NIK, IKK-α and IKK-β. As shown in Figs. 2A and 2B, 5-FU did not affect the expression levels of IKK-α and IKK-β proteins. Thus, we attempted to analyze the IKK activity in cl-1 cells stimulated with 5-FU. Following immunoprecipitation of the IKK-α/IKK-β heterodimer (20) from cell extracts using an anti-IKK-β antibody, IKK activity was determined by phosphorylation of the substrate GST-IκB-α. As shown in Fig. 2C, IKK activity in cl-1 cells was significantly inhibited at both 6 and 12 h after treatment with 5-FU.

Effect of Concentration of 5-FU on the Suppression of IKK Activity

To determine the concentration of 5-FU that would be most effective for suppressing IKK activity, we treated cl-1 cells with 5-FU at concentrations ranging

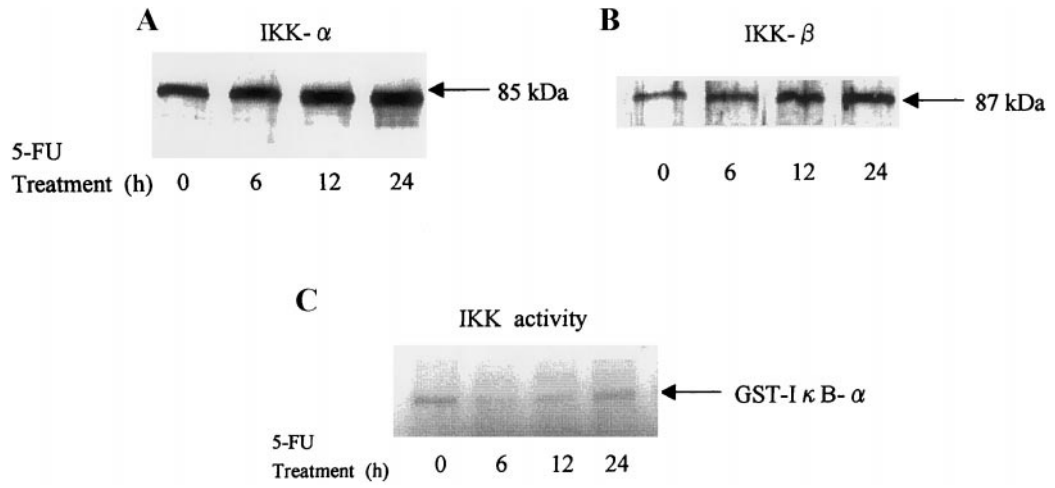

FIG. 2. Western blot analysis for the expression of IKK-α (A) and IKK-β (B) proteins in cl-1 cells. Cytoplasmic extracts were prepared from cells following 5-FU (2 μg/ml) treatment for the indicated time points. 5-FU treatment did not affect the expression levels of IKK-α or IKK-β proteins. C) *In vitro* kinase assay to determine the effect of 5-FU on the IKK activity in cl-1 cells. Cell lysates obtained after 5-FU treatment for the indicated time points were immunoprecipitated with an anti-IKK-β antibody. Kinase assays contained GST-IκB-α as a substrate. IKK activity was suppressed in cl-1 cells at 6 and 12 h after treatment with 5-FU.

from 1 to 10 μg/ml. As can be seen in Fig. 3A, it was evident that 2 μg/ml of 5-FU most effectively inhibited IKK activity. However, the expression levels of IKK-α and IKK-β proteins were constitutively detected even after treatment with 5-FU (Figs. 3B and 3C).

Effect of 5-FU on the Expression of FLIP

Since the activity of caspase-8 is regulated by FLIP (21), we examined the kinetics of FLIP expression in 5-FU-treated cells. As shown in Fig. 4, 5-FU suppressed the expression of FLIP protein at both 6 and 12 h after treatment.

Cleavage of PARP by 5-FU

PARP is a 116 kDa nuclear enzyme that detects and binds DNA strand breaks produced by various apoptotic stimuli, and it is a known substrate for caspase-3 (22). Caspase-3-mediated PARP cleavage into its molecular weight of 85 kDa is considered to be one of the hallmarks of apoptosis (23). Thus, since we previously showed that 5-FU activates caspase-3 in cl-1 cells (16), we investigated the cleavage of PARP by 5-FU in the present study. As shown in Fig. 5, increased cleavage of PARP was observed for up to 24 h after treatment with 5-FU.

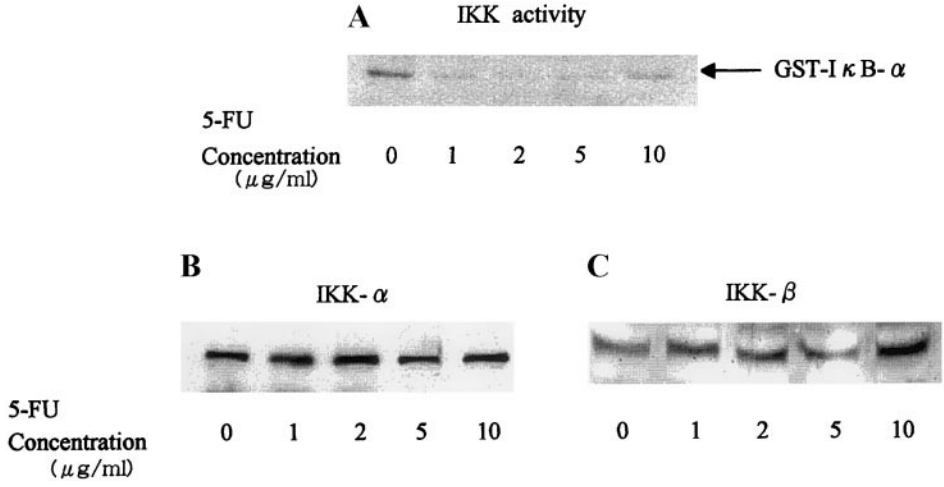

FIG. 3. (A) *In vitro* kinase assay for determination of the effective dose of 5-FU in the suppression of IKK activity in cl-1 cells. Cells were treated with various concentrations of 5-FU for 12 h, and cell lysates were obtained. 5-FU at the concentration of 2 μg/ml most effectively suppressed the IKK activity in cl-1 cells. (B and C) Western blot analysis for the expression of IKK-α (B) and IKK-β (C) proteins in cl-1 cells after treatment with various concentrations of 5-FU for 12 h. 5-FU did not affect the expression levels of IKK-α or IKK-β proteins.

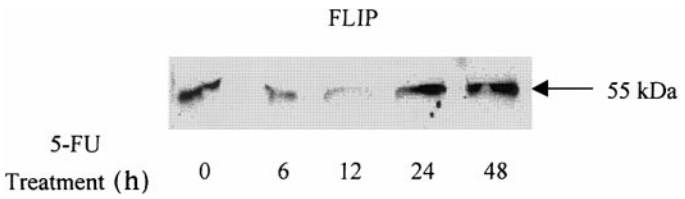

FIG. 4. Effect of 5-FU on the expression of FLIP protein in cl-1 cells. Western blot analysis demonstrated that expression levels of FLIP protein were inhibited at 6 and 12 h after treatment with 5-FU (2 μg/ml).

DISCUSSION

Inhibitors of thymidylate metabolism represent an important class of antineoplastic agents used for the treatment of head and neck cancers. Clinical response to 5-FU-based regimens is typically between 20 and 30%, and drug resistance, either intrinsic or acquired, is a common phenomenon (24). Because response is difficult to predict using the TNM classification system (proposed by the International Union Against Cancer) alone, considerable effort has been directed toward understanding the mechanisms of drug action. Thus, to date, two different mechanisms have been identified regarding the action of 5-FU as follows: (a) the inhibition of the enzyme thymidylate synthase by the anabolite 5-fluoro-dUMP; and b) the interference of maturation of rRNA because of the incorporation of 5-fluoro-UTP (25). In addition to the above mechanisms, we have recently reported a novel pathway in which 5-FU induces apoptosis in cancer cells, i.e., 5-FU suppresses NF-κB activity in cl-1 cells, followed by the activation of caspase-8 and caspase-3 (16). However, the mechanism underlying the suppression of NF-κB activity by 5-FU has not been elucidated. Thus, in this study, we focused our interest not only on the mechanism involved in the prevention of degradation of IκB-α by 5-FU, but also on the expression of FLIP, a caspase-8 inhibitor, and the cleavage of PARP, a target of caspase-3. As a consequence, we demonstrated here that 5-FU prevented the degradation of IκB-α protein by suppressing the IKK activity in cl-1 cells and that inhibition of FLIP expression and the cleavage of PARP were observed as a result of 5-FU treatment. Since we have recently shown that several human head and neck carcinoma cell lines express high NF-κB activity owing to the enhanced IKK activity (submitted for publication), 5-FU would be one of the most suitable chemotherapeutic agents for the treatment of patients with head and neck cancer.

Biochemical analysis indicates that the predominant form of IKK is an IKK-α/IKK-β heterodimer (26) and that both kinases make essential contributions to IκB-α phosphorylation and NF-κB activation (20). Therefore, we analyzed the total IKK activity (IKK-α and IKK-β) using an immunoprecipitation technique. *In vitro* kinase assay revealed that IKK activity was maximally inhibited by 5-FU at a concentration of 2 μg/ml. Since this concentration of 5-FU is the pharmacologic concentration in the serum of patients that is necessary for the induction of anti-cancer properties (19), our result in the *in vitro* kinase assay strengthens the case for the clinical usefulness of 5-FU at the serum concentration of 2 μg/ml. Although we did not examine the mechanism involved in the suppression of IKK activity by 5-FU, it has been reported that the anti-inflammatory agents salicylate and aspirin inhibit the NF-κB pathway by direct binding of aspirin and salicylate to IKK resulting in their competition for its binding to ATP (27).

FLIP has been shown to render cells resistant to apoptotic signals transmitted by all death receptors known to date by inhibition of caspase-8 recruitment to the death receptor complex (28). In addition, up-regulation of FLIP expression may be involved in the pathogenesis of tumors, such as melanomas, because FLIP was found to be overexpressed in malignant melanomas, whereas no FLIP expression was detected in normal melanocytes (28). Thus, FLIP may play an important role in tumorigenesis and in the modulation of sensitivity or resistance to cancer chemotherapy. In our present study, 5-FU down-regulated the expression of FLIP protein in cl-1 cells, leading to the stimulation of caspase-8 activity by 5-FU (16).

The function of PARP is thought to be related to a number of nuclear processes that involve the nicking and resealing of DNA strands, including transcription and DNA repair (22), and the cleavage of PARP mediated by caspase-3, a key executioner in the DNA fragmentation process and other morphological changes (30, 31), is one of the characteristics of apoptosis. Since PARP with a molecular weight of 116 kDa is cloven by caspase-3 into its molecular weight of 85 kDa, we examined the kinetics of cleavage of PARP by 5-FU and showed that PARP was actually cloven in cl-1 cells. This result, taken together with those of our previous study (16), indicates that 5-FU exerts its apoptotic function on cancer cells by cleaving PARP through the activation of caspase-3.

In conclusion, the results of this study suggest that a novel molecular mechanism is involved in the induc-

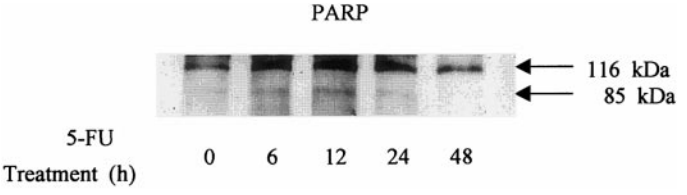

FIG. 5. 5-FU induces PARP cleavage in cl-1 cells. Cell lysates were subjected to Western blot analysis. PARP with a molecular weight of 116 kDa was cloven into a lower molecular weight species (85 kDa).

tion of apoptosis by 5-FU. Because chemotherapeutic agents exert their cytotoxic effects through several distinct pathways that function additively or synergistically, the mechanism of 5-FU action analyzed in this study seems to be one of the mechanisms underlying the induction of apoptosis in cancer cells.

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