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5-Fluorouracil inhibits nitric oxide production through the inactivation of IκB kinase in stomach cancer cells

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Abstract

The antimetabolite 5-fluorouracil (5-FU) is one of the more prominent clinical antitumor agents available for the treatment of stomach and colorectal cancers. In the present study, we characterized the effects of 5-FU on nitric oxide (NO) production by cells from the stomach cancer cell line NCI-N87. A cytokine mixture [interleukin (IL)-1β/interferon (IFN)-γ] increased the production of NO by stomach cancer cells in a concentration- and time-dependent manner. Pretreatment with 5-FU inhibited the production of NO that was stimulated by the cytokine mixture and reduced the expression of iNOS. The cytokine mixture activated nuclear factor κB (NF-κB) in a concentration- and time-dependent manner, which was blocked by 5-FU pretreatment. The pretreatment with 5-FU stabilized IκBα and inactivated IκB kinase. Collectively, these data suggest that the efficacy of 5-FU may include the inactivation of IκB kinase and the inhibition of NO production.

Keywords: Stomach cancer; 5-Fluorouracil; Nitric oxide; NF-κB; IκBα

1. Introduction

NO is a short-lived free radical synthesized from arginine with extremely high reactivity and a variety of physiological activities [1]. NO is synthesized by a family of three distinctive NOS isoforms named for the tissues in which they were originally described. Neuronal type NOS (nNOS) and endothelial type NOS (ecNOS) are calcium-dependent and constitutively express relatively low amounts of NO. An inducible calcium-independent form, iNOS, may be found in macrophages, hepatocytes, neutrophils, endothelial cells, and astrocytes. NO causes DNA damage and is a potential endogenous carcinogen [2]. Increased NO production may increase angiogenesis and contribute to tumor progression [3]. However, the effects of NO on tumor cells are apparently output-dependent and cell-type specific [4]. Overproduction of NO is cytotoxic, induces apoptosis, and suppresses tumor growth, whereas a low output of NO may protect cells from apoptosis and promote tumor growth [5].

Since its introduction over 35 years ago, 5-FU has been one of the most effective chemotherapeutic options available for the treatment of advanced colorectal and stomach cancers. Jin *et al.* [6] reported that this antimetabolite inhibited the production of NO, which was not related to the incorporation of 5-FU into the iNOS mRNA, but did not identify the precise mechanism by which it inhibits this production. 5-FU has also been reported to induce apoptosis by suppression of NF-κB via inhibition of IκB kinase activity in human salivary gland cancer cells [7]. However, there was no explanation of the function of NO on 5-FU-induced cytotoxicity. In the present study, we demonstrated that 5-FU inhibits the production of NO by inactivating NF-κB through the stabilization of IκB in human stomach cancer cells.

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Abbreviations: CM, cytokine mixture; EMSA, electrophoretic mobility shift assay; 5-FU, 5-fluorouracil; IκB, inhibitor κBα; IFN-γ, interferon-gamma; IL-1β, interleukin-1 beta; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; NF-κB, nuclear factor κB; RT–PCR, reverse transcription–polymerase chain reaction.

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2. Materials and methods

2.1. Chemicals

3-(4,5-Dimethyl-thiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), IFN-γ, and 5-FU were purchased from the Sigma Chemical Co. IL-1β was obtained from Roche Diagnostics GmbH, and TriZol from Life Technologies, Inc. Radioactive materials were obtained from the Amersham Corp.

2.2. Cell culture

The stomach cancer cell line NCI-N87 was obtained from the Korean Cell Line Bank and grown at 37°, 5% CO_2 in RPMI 1640 medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum. Stomach cancer cells (2×10^6/mL) were dispensed on 60-mm culture dishes and stimulated with CM (200 U/mL of IFN-γ and 1 ng/mL of IL-1β) in the presence or absence of 5-FU at the indicated concentrations and time points. NO production was quantified by measuring nitrite plus nitrate (NO_2^- and NO_3^-) by an automated procedure based on the Griess assay [8].

2.3. RT–PCR amplification

Total RNA was isolated using TriZol (Life Technologies, Inc.), following the suggested protocol of the manufacturer. Using 5 μg of total RNA, RT–PCR analysis was performed as described [9]. To make specific probes of human iNOS and β-actin, RT–PCR reactions were performed using human iNOS primers 5′-CCCGAGTCAGAGTCACCATCC-3′ and 5′-TCAAACGTCTCACAGGCTGCC-3′, and human β-actin primers 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ and 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′. The band density was analyzed utilizing EagleSight Software v. 3.2 (Stratagene).

2.4. EMSA

Adherent cells (5×10^6/100-mm tissue culture dish) were stimulated with CM in the presence or absence of 5-FU, washed, and scraped into 1 mL of cold phosphate-buffered saline. The cell suspensions were transferred to microcentrifuge tubes and pelleted, and the nuclear protein extracts were prepared essentially as described [10]. The nuclear extracts were centrifuged at 19,300 *g* for 30 min at 4°, and the supernatants were frozen at −70° in aliquots until EMSAs were done. Protein was quantified by the Bradford assay (Bio-Rad). EMSAs were performed by incubating ^{32}P-labeled NF-κB consensus oligonucleotide (5′-AGC TTG GGG ACT TTC C-3′) with 5 μg of nuclear extracts as described previously [10].

2.5. IκB kinase activity

After pretreatment with or without 5-FU for 12 hr, NCI-N87 cells were incubated further with the CM for 5 min. Cell lysates were mixed with 5 μg of a rabbit affinity-purified anti-IκB kinase 2 polyclonal antibody (Santa Cruz Biotechnology). The immunoprecipitate was prepared as described previously [11], resuspended in kinase buffer, and incubated at 30° for 30 min in the presence of the substrate GST-IκBα (1–54). Proteins were separated by SDS–PAGE, and radiolabeled proteins were visualized by autoradiography.

2.6. Western blot analysis

Control and treated NCI-N87 cells were first lysed in buffer containing 30 mM Tris–HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/mL of leupeptin, 5 μg/mL of pepstatin, and 5 μg/mL of trypsin inhibitor. Cell extracts were then fractionated on SDS–polyacrylamide gels, transferred to nitrocellulose, and analyzed with polyclonal antibodies to iNOS (Transduction Laboratories), IκBα and p65 (Santa Cruz Biotechnology), and visualized by the ECL-system (Amersham) using anti-rabbit horseradish peroxidase IgG (Sigma). The band density was analyzed utilizing EagleSight Software v. 3.2.

2.7. Measurement of cell viability

Cell viability was measured by tetrazolium reduction using the MTT assay [12]. After each experiment, drug-treated samples showing higher than 85% of the nontreated control were used.

2.8. Statistical analysis

Results are expressed as means ± SD, and an analysis was done by Student's one-way *t*-test. *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Effects of CM on the production of NO in stomach cancer cells

Reactive oxygen and nitrogen species have been proposed to play an important role in human carcinogenesis. Many cancer cells including cholangiocarcinoma and colon cancer cells have a cytokine-induced NOS and produce an increased amount of NO in the presence of the CM [13–15]. Although human stomach cancer cells contain iNOS and its increased activity has been observed in patients with chronic gastritis and gastric cancer [16,17], the inducibility of iNOS in human stomach cancer cells by

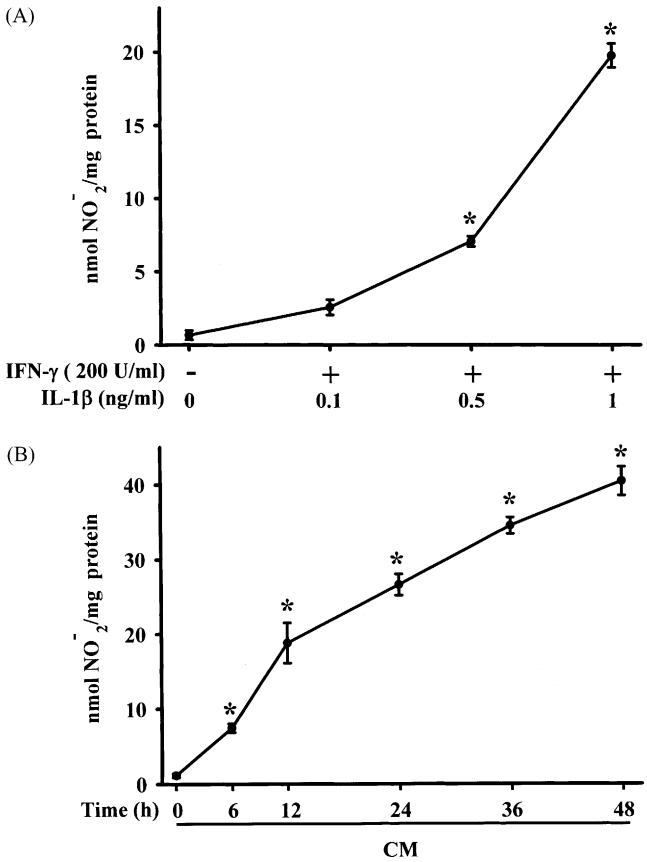

Fig. 1. Effects of the CM on the production of NO by NCI-N87 cells. NCI-N87 stomach adenocarcinoma cells were treated with the CM, and NO production was quantified by measuring the accumulation of nitrite in the culture medium as indicated in Section 2. (A) Concentration-dependent increase in NO production 24 hr after treatment with 200 U/mL of IFN-γ and various amounts of IL-1β. (B) Time course of nitrite accumulation with 200 U/mL of IFN-γ and 1 ng/mL of IL-1β. Data are the means ± SD from triplicate samples. Key: (*) $P < 0.05$, significantly different from control.

cytokines like IL-1β/IFN-γ has not been examined. To verify whether this CM can stimulate the production of NO by stomach cancer cells, we treated NCI-N87 cells with the CM and found that it increased the production of NO in a concentration- and time-dependent manner (Fig. 1A and B). These data show that iNOS in human stomach cancer cells is activated by this CM.

3.2. Effects of 5-FU on the production of NO in response to the CM

We next examined the effects of 5-FU on NO production by NCI-N87 cells stimulated with the CM. The antimetabolite 5-FU is one of the most prominent clinical antitumor agents that display significant activity toward colorectal and stomach cancers. Recently, Jin *et al.* [6] reported that 5-FU inhibited the production of NO by colorectal cancer cells (DLD-1) stimulated with IFN-γ. Furthermore, this inhibition of NO production by 5-FU was not reversed by the addition of thymidine to the colon cancer cells. Since 5-FU is widely used for the treatment of stomach cancer without knowing the precise mechanism how it inhibits tumor growth, we tested whether this anticancer agent shows the same effect on NO production in stomach cancer cells as it does in colon cancer cells. Stomach cancer cells were pretreated with 5-FU for 12 hr and further incubated with the CM for another 12 hr to stimulate NO production. 5-FU inhibited the production of NO by NCI-N87 cells in a concentration-dependent manner, with an IC_{50} value of 25.2 μM (Fig. 2A). 5-FU alone had no effect on NO production by the cells, and no cytotoxicity was observed with the concentrations we tested (Fig. 2A and B). We then compared the inhibition level of 5-FU on NO production with a specific competitive NOS inhibitor, L-NAME. 5-FU at 40 μM inhibited the production of NO by the CM more extensively than did L-NAME at 1 mM (Fig. 2C). These data strongly suggest that the CM-induced increase in NO production could be blocked by 5-FU pretreatment without any cytotoxicity.

3.3. Effects of 5-FU on the expression of NOS

In mammalian cells, NO is generated by the NADPH-dependent oxidation of arginine to citrulline by NOS. Therefore, we tested whether the inhibition of NO production by 5-FU in response to the CM is caused by its effect on iNOS expression. The increase in iNOS mRNA production in the presence of the CM was time-dependent (Fig. 3A), and preincubation with 5-FU for 12 hr before CM treatment reduced iNOS dramatically (Fig. 3B). However, the expression of ecNOS was not affected by the 5-FU treatment (data not shown). These results suggest that the effects of 5-FU on the production of NO by stomach cancer cells are either due to interference with the transcription of iNOS DNA or the promotion of iNOS mRNA degradation. However, our unpublished data showed that 5-FU did not affect the stability of iNOS mRNA. Therefore, it is highly possible that 5-FU inhibits iNOS mRNA expression in stomach cancer cells in response to CM activation.

3.4. Effects of 5-FU on the activation of NF-κB stimulated by the CM

NF-κB is critical for the inducible expression of many genes involved in inflammation [18]. It is a dimeric transcription factor composed of homodimers and heterodimers of Rel proteins, of which there are five family members in mammals [i.e. RelA (p65), C-Rel, RelB, NF-κB1 (p50), and NF-κB2 (p52)] [19]. Since the CM was reported to induce human iNOS transcription through the NF-κB site [13,20], and our study showed that the CM increases the production of NO by stomach cancer cells, we first treated the stomach cancer cells with 200 U/mL of IFN-γ and increasing amounts of IL-1β to identify the effects on NF-κB activation. As shown in panels A and B of Fig. 4, the CM activates NF-κB in a concentration- and

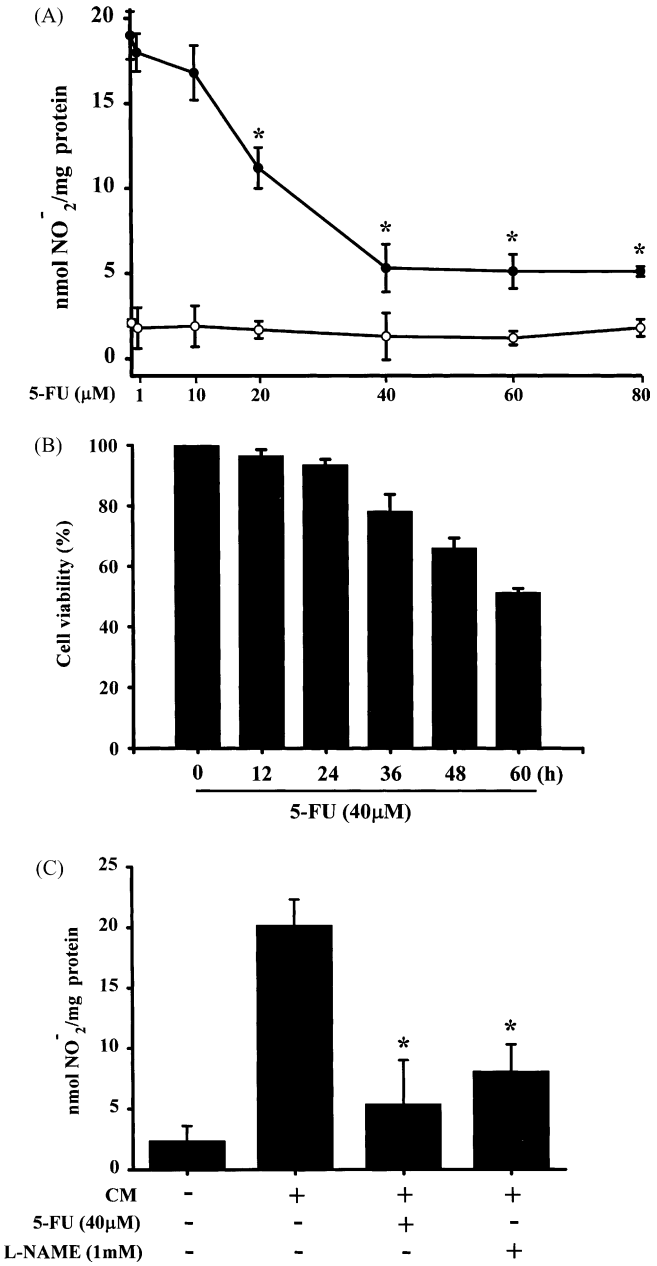

Fig. 2. Effects of 5-FU on the production of NO by NCI-N87 cells. (A) After incubation with various concentrations of 5-FU for 12 hr, stomach cancer cells were treated with (●) or without (○) the CM for another 12 hr. (B) Cytotoxicity assay. Stomach cancer cells were incubated for the indicated times with 5-FU (40 μM), and cell viability was measured by using the MTT assay as described in Section 2. (C) After incubation with 5-FU (40 μM) or L-NAME (1 mM), an NOS inhibitor, for 12 hr, cancer cells were treated with the CM for another 12 hr and NO production was quantified by measuring the accumulation of nitrite in the culture medium. Data are the means ± SD from triplicate samples. Key: (*) $P < 0.05$, significantly different from control.

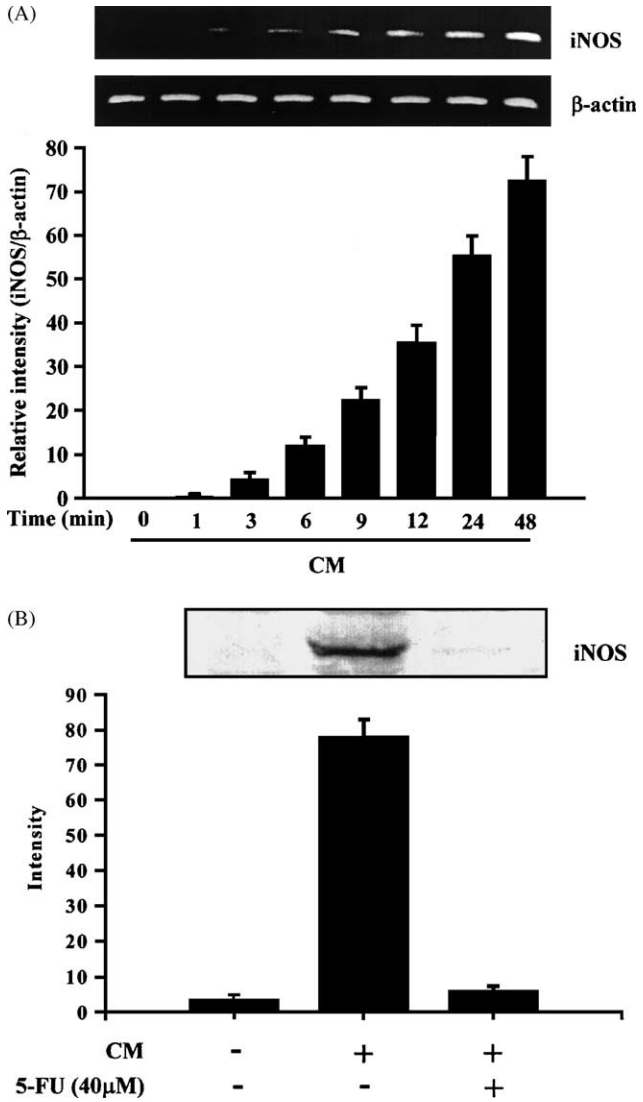

Fig. 3. Effects of 5-FU on the expression of iNOS in NCI-N87 cells. (A) Cells were treated with the CM for the times indicated, and iNOS mRNA expression was measured by RT–PCR using specific iNOS primers. (B) Western blot analysis. Cell extracts (25 μg) were fractionated on an SDS–polyacrylamide gel and analyzed with polyclonal antibodies to iNOS. The band intensity was analyzed utilizing EagleSight software v. 3.2. The data shown are a typical representation of four separate experiments with three replicates.

time-dependent manner. To investigate whether 5-FU inhibits the activation of NF-κB by the CM in stomach cancer cells, we pretreated the NCI-N87 cells with 5-FU and further incubated them with the CM. Nuclear extracts were examined by EMSA for NF-κB binding activity using consensus NF-κB binding sequences. Fig. 4C shows that the increase in NF-κB binding activity induced by the CM is blocked by pretreatment with 5-FU in a concentration-dependent manner. Taken together, these data suggest that the CM activates NF-κB and pretreatment with 5-FU inhibits this activation.

3.5. Effects of 5-FU on the stabilization of IκBα and on the activation of IκB kinase by the CM

NF-κB dimers are held in the cytoplasm in an inactive state by inhibitory proteins, the IκBs that preferentially associate with various Rel family protein dimers [21]. Active NF-κB is released from the cytoplasmic complex and enters the nucleus via an IκB kinase-catalyzed phos-

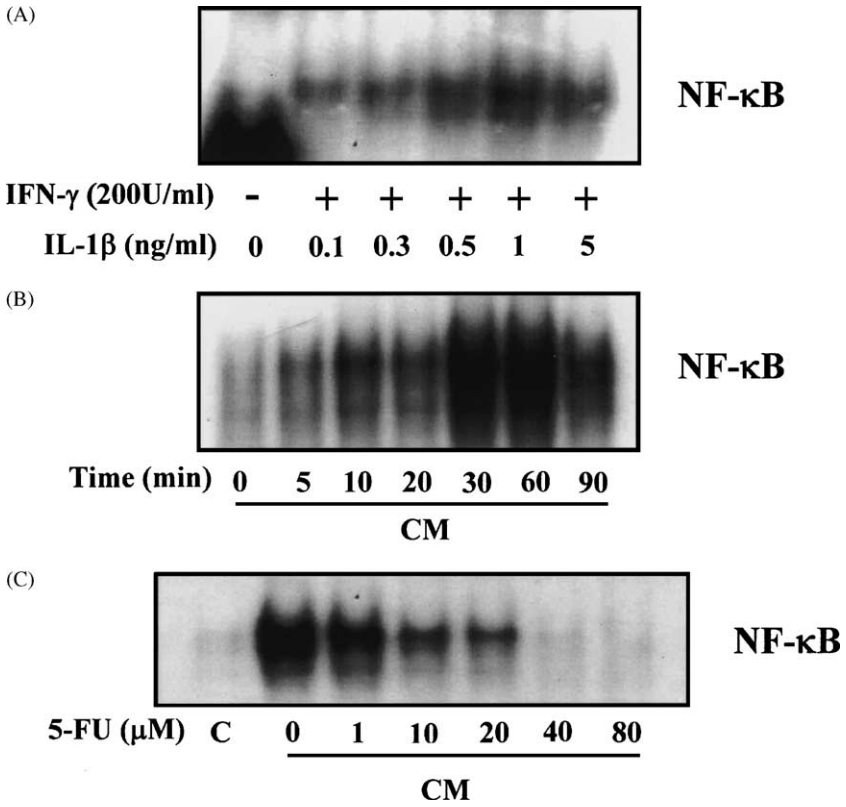

Fig. 4. Effects of 5-FU on the activation of NF-κB by the CM. (A) Stomach cancer cells were incubated with or without 200 U/mL of IFN-γ and various concentrations of IL-1β for 1 hr. (B) Cancer cells were incubated with or without the CM for the indicated times. (C) Cancer cells were pretreated with various concentrations of 5-FU for 12 hr and further incubated with the CM for 1 hr. Isolation of nuclear extracts and EMSA analysis were described in Section 2. The data shown are a typical representation of three separate experiments.

phorylation and the proteolytic degradation of IκBα, by the 26S proteasome in response to various cytokines, to unmask NF-κB nuclear translocation sequences [22]. Since 5-FU inhibits the activation of NF-κB by the CM in stomach cancer cells, we investigated whether 5-FU causes the stabilization of IκBα and prevents the translocation of p65 to the nucleus. Treatment with the CM led to a rapid disappearance of the immunoreactive IκBα band within 10 min; this protein returned to basal levels within 30 min (Fig. 5A). Maximum p65 translocation into the nucleus was observed within 20 min of CM treatment. To identify the effects of 5-FU on the stability of IκBα and p65 translocation, cells were pretreated with 5-FU at 40 μM for 12 hr and further incubated with the CM. As shown in Fig. 5B, 5-FU protected IκBα from proteolysis by the 26S proteasome and decreased the amount of p65 in the nucleus in a concentration-dependent manner, indicating that 5-FU inhibits NO production through the stabilization of IκBα. IκBα is phosphorylated by IκB kinase on Ser^{32} and Ser^{36} to be a target for ubiquitination and subsequent degradation by the proteasome [11,23–26]. Fig. 5C shows that the CM activates IκB kinase to increase the phosphorylation of IκBα. However, pretreatment with 5-FU decreased IκB kinase activation by the CM in a concentration-dependent manner (Fig. 5D), suggesting that the inhibition of NO production by 5-FU in response to the CM might be caused by its inactivation of IκB kinase. These results are consistent with the effects of 5-FU in human salivary gland cancer cells [7]. 5-FU inactivated IκB kinase while the expression of IκB kinase was not affected. At present, we do not know how 5-FU suppresses IκB kinase. However, it is possible that 5-FU binds directly to IκB kinase in similar fashion to anti-inflammatory agents such as aspirin and salicylate to inhibit the NF-κB pathway [27].

The function of NO in tumor development, promotion, and progression is not clear. One can speculate that NO plays both beneficial and detrimental roles in patients with stomach cancer. On the one hand, it has been shown to be responsible for the growth arrest and terminal differentiation of cells, but it could also promote tumor growth through angiogenesis. Jenkins *et al.* [28] demonstrated that growth, invasion, and metastasis of colon tumors were enhanced by NOS expression. Furthermore, activation of iNOS and excessive production of NO in response to inflammatory cytokines cause DNA damage and inhibit DNA repair proteins [29]. iNOS and ecNOS have been detected in stomach cancer tissue [17]. However, little is known about the effect of NO on tumor growth and the metastasis of stomach cancer. Our study strongly suggests that the production of NO by stomach cancer cells is inhibited in the presence of 5-FU through the inactivation of IκB kinase. Further work on iNOS overproduction in

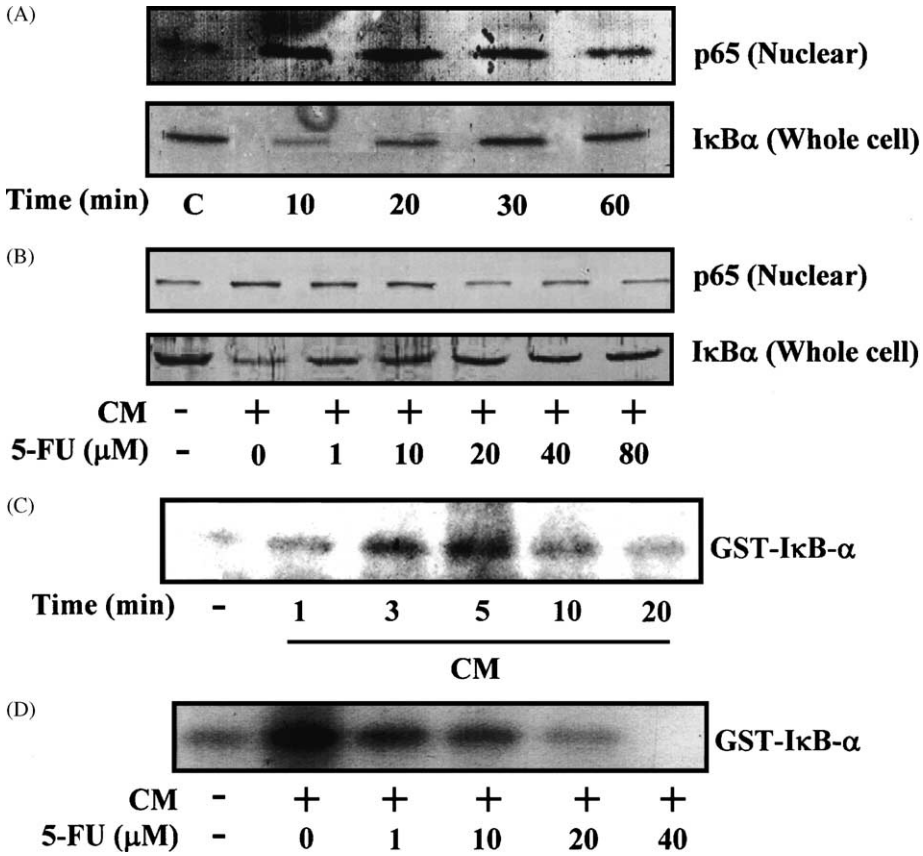

Fig. 5. Effects of 5-FU on IκBα stability and IκB kinase activation by the CM. (A) Time course of p65 and IκBα. Cells were incubated with the CM for the indicated time points, and nuclear (p65) or whole cell (IκBα) extracts were analyzed by western blotting with each specific polyclonal antibody, respectively. (B) Translocation of p65 to the nucleus and stability of IκBα. After treating with various concentrations of 5-FU for 12 hr, cells were incubated further with the CM for 20 min (p65), or 10 min (IκBα), and nuclear (p65) or whole cell (IκBα) extracts (25 μg each) were fractionated in 10% SDS–PAGE. (C) Activation of IκB kinase. Cancer cells were incubated with the CM for the indicated time points. (D) Effect of 5-FU on IκB kinase activity. Cancer cells were pretreated with 5-FU for 12 hr, further incubated with the CM for 5 min, and cell extracts prepared. IκB kinase was immunoprecipitated from cell extracts, and kinase activity was determined using GST-IκBα (1–54) as substrate. The data shown are a typical representation of three separate experiments.

stomach cancer cells and its effects on the growth and metastasis of stomach cancer cells will produce valuable data about the use of 5-FU in stomach and colorectal cancer patients.

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