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Gefitinib (Iressa, ZD1839) inhibits SN38-triggered EGF signals and IL-8 production in gastric cancer cells

Abstract Epidermal growth factor receptor (EGFR) and its ligands are involved in tumor growth, metastasis, angiogenesis, and resistance to chemotherapy. In the experiments described here using AGS gastric cancer cells, SN38 (the active metabolite of CPT-11) induced tyrosine phosphorylation of EGFR within 5 min, and this was followed by the induction of transcripts and/or proteins of heparin-binding EGF-like growth factor, amphiregulin, transforming growth factor-α, and interlukin-8 (IL-8). SN38 also activates nuclear factor-κB and activator protein-1, both of which are critical for the transcription of the IL-8 gene. However, the blocking of EGFR activation by gefitinib (Iressa, ZD1839), an EGFR-TKI (tyrosine kinase inhibitor), abrogates all the above reactions. The SN38-triggered mechanisms include the generation of reactive oxygen species (ROS) and the activation of protein kinase C (PKC), followed by metalloproteinase activation and the sequential ectodomain shedding of EGFR ligands. These findings suggest that EGF signaling is enhanced by CPT-11 and point to the potential benefit of the use of a combination of CPT-11 with gefitinib in the treatment of certain gastric cancers.

Keywords Gefitinib · EGF · EGF receptor · SN38 · IL-8

Abbreviations AP-1: Activator protein-1 · AR: Amphiregulin · CPT,: Camptothecin · EGF: Epidermal growth factor · EGFR: EGF receptor · ELISA:

Enzyme-liked immunosorbent assay · EMSA: Electrophoretic mobility shift assay · GAPDH: Glyceraldehyde 3-phosphate dehydrogenase · HB-EGF: Heparin-binding EGF-like growth factor · IL-8: Interleukin-8 · NAC: N-acetylcysteine · NF-κB: Nuclear factor-kappaB · ROS: Reactive oxygen species · TGF: Transforming growth factor · TKI: Tyrosine kinase inhibitor

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Introduction

Camptothecin (CPT), a DNA topoisomerase inhibitor, blocks DNA religation of topoisomerase I cleavage complexes, leading to DNA damage [1]. Irinotecan hydrochloride (CPT-11) is a water-soluble semisynthetic derivative of CPT that retains the original antitumor activity of CPT but is less toxic [2]. Although CPT-11 is currently in clinical use for the treatment of many types of solid tumors including lung, colorectal, ovarian, uterine, and gastric carcinomas [3, 4], the response rate to CPT-11 alone is not always high because of various factors, including the loss of p53, the presence of the DNA mismatch repair gene *hMLH1*, and the overexpression of the *Drg1* gene which modulates CPT-11 sensitivity [5].

It has been reported that several genotoxic drugs are capable of producing reactive oxygen species (ROS) in cancer cells [6]. Levels of ROS above normal are referred to as a state of oxidative stress, which may contribute to cell death, partly through modulating the levels of expression of several apoptosis-related genes. We have recently found that oxidative stress paradoxically induces rapid tyrosine phosphorylation of epidermal growth factor receptor (EGFR), followed by a marked induction of EGF-like growth factors in a gastric epithelial cell line [7]. Moreover, another study has shown that the tumor-promoting activity associated with oxidative stress is mediated mainly through the activation of EGFR [8]. Though the precise mechanism by which

ROS transactivate EGFR is unclear, it is widely recognized that ROS rapidly mediate EGFR phosphorylation through a ligand-independent mechanism. On the other hand, the ectodomain shedding of EGFR ligands including EGF, transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin (AR), generate soluble forms which act in an autocrine or paracrine fashion, and transmembrane forms which act in a juxtacrine fashion [9]. These ligands activate EGFR via a ligand-dependent mechanism.

Recent studies have shown that a variety of diverse metalloproteinase disintegrins are involved in ectodomain shedding and, moreover, different stimuli can induce ectodomain shedding of an individual growth factor precursor via different metalloproteinases [9]. Since EGF signals are involved in the regulation of a variety of cell functions including cell growth, migration, survival, and angiogenesis [10], the activation of EGFR may be involved in chemoresistance in solid tumors [11]. However, the effect of genotoxic drugs, including CPT-11, on activation of EGFR and subsequent EGFR-mediated transcriptional responses is unknown.

A large body of experimental and clinical data supports the concept that EGFR is a relevant target in cancer therapy. Different pharmacological and biological approaches have been developed for blocking EGFR activation and/or its function in cancer cells [11, 12]. Gefitinib (Iressa, ZD1839) is an orally active quinazoline-derived agent that inhibits EGFR-tyrosine kinase [12, 13]. Previous preclinical studies have demonstrated an enhancement of the antitumor activity of ionizing radiation and genotoxic drugs following treatment with gefitinib [14, 15]. In addition, a recent study has shown that gefitinib reverses resistance to irinotecan and enhances its efficacy by improving its oral bioavailability in colorectal cancer [16].

The aim of the present study was to examine whether SN38 (the active metabolite of CPT-11) activates EGFR and induces EGFR-mediated transcriptional responses in gastric cancer cells. For this purpose, we determined the effect of SN38 on tyrosine phosphorylation of EGFR, expression of EGF-like ligands, activation of activator protein-1 (AP-1) and nuclear factor-kappaB (NF-κB), and induction of interleukin-8 (IL-8) in gastric cancer cell lines.

Materials and methods

Reagents

SN38 and gefitinib were donated by Yakult Honsha (Tokyo, Japan) and AstraZeneca Corporation (San Diego, Calif.). N-Acetyl-L-cysteine (NAC), genotoxic drugs, except SN38, and the ROS-sensitive fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate diacetoxymethyl-ester (H$_2$-DCFDA) were purchased from Sigma-Aldrich (St. Louis, Mo.). The anti-human EGF receptor antibody (clone LA22) was purchased from Upstate Biotechnology (Lake Placid, N.Y.). The rabbit anti-ErbB-2 (Neu) antibody, rabbit anti-ErbB-3 antibody, and mouse anti-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). The rabbit anti-phospho-pan, $\alpha\beta$, δ, θ, ζ/λ-PKC antibody, mouse anti-phosphotyrosine monoclonal antibody (p-thy-100), HRP-linked anti-mouse IgG and HRP-linked anti-rabbit IgG were purchased from Cell Signaling Technology (Beverly, Mass.). The mouse anti-Integrin β1 antibody was purchased from BD Biosciences (San Jose, Calif.).

Cell lines

The human gastric adenocarcinoma cell line AGS (ATCC CRL 1739) was cultured in Ham's F-12 medium (Nacalai Tesque, Kyoto, Japan). MKN28, MKN45, and MKN74 cell lines were cultured in RPMI1640 medium (Nacalai Tesque). These cell lines were supplemented with 10% heat-inactivated fetal bovine serum (Bio-Whittaker, Walkersville, Md.), and 100 U/ml each of penicillin and streptomycin in a water-saturated atmosphere containing 5% CO$_2$ at 37°C.

Immunoprecipitation and western blots

Cells were lysed in 1 ml ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate-Na, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, 10 μg/ml leupeptin, and 2 mM Na$_3$VO$_4$; pH 8.0) and clarified by centrifugation at 15,000 rpm for 15 min. The supernatant was incubated overnight at 4°C with an anti-human EGF receptor antibody coupled to protein G-Sepharose (Amersham, Little Chalfont, UK). The precipitates were boiled in SDS sample buffer for 2 min, and electrophoresed on an SDS gradient gel (ATTO, Tokyo, Japan). The proteins were transferred to nitrocellulose membranes (Millipore Corporation, Bedford, Mass.). The membranes were blocked for 60 min at room temperature in blocking buffers, then washed with TBS/Tween and incubated with the appropriate antibody overnight at 4°C. After removing the primary antibody, the blots were placed in the appropriate HRP-conjugated secondary antibody for 60 min. Proteins were then detected using an enhanced chemiluminescence system (ECL, Amersham). In some experiments, the membrane were then stripped and reprobed with the appropriate antibody as an internal control to verify equivalency of protein loadings.

Evaluation of intracellular ROS

Intracellular ROS levels were estimated using an oxidant-sensitive H$_2$-DCFDA probe. Cells were grown in
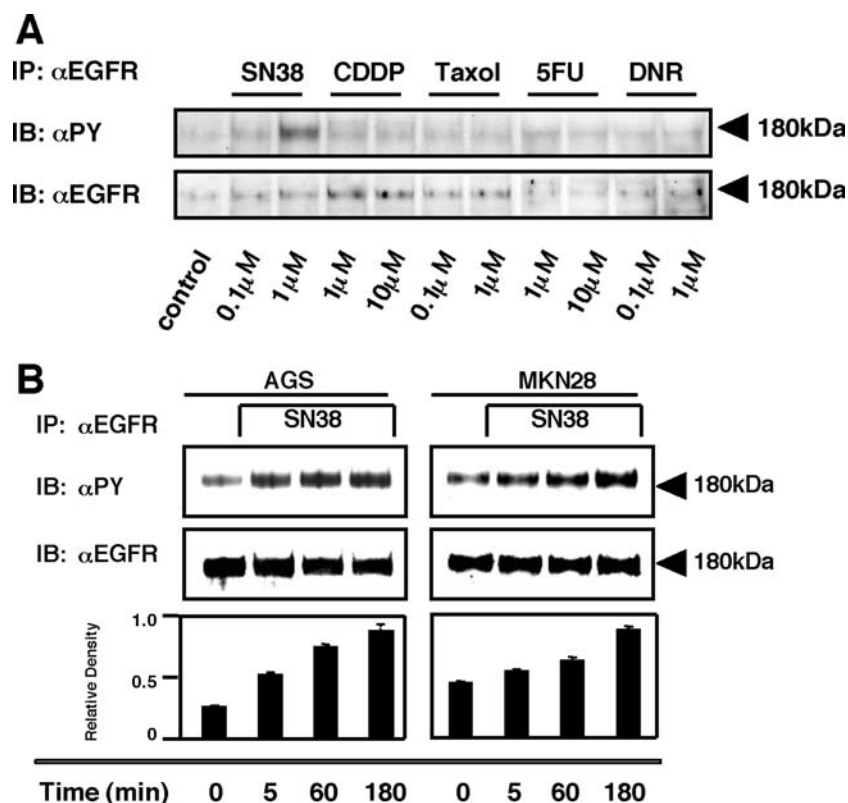
Fig. 1 a SN38-induced tyrosine phosphorylation of EGF receptor in AGS cells. AGS cells were incubated with SN38, cisplatin (*CDDP*), paclitaxel (*Taxol*), 5-fluorouracil (*5-FU*), and daunorubicin (*DNR*) for 120 min at the indicated concentrations. Thereafter, the cells were lysed, and immunoprecipitation was performed using the anti-EGF receptor antibody. The precipitates were separated by SDS-PAGE and transferred to membranes. Immunoblots were probed with an anti-phosphotyrosine antibody (αPY). Filters were subsequently reprobed with an anti-EGF receptor antibody (αEGFR). Each figure shows representative data from four similar experiments. **b** Time-course of SN38-induced tyrosine phosphorylation of EGFR in AGS and MKN28 cells. Cells of each cell type were incubated with 40 ng/ml SN38 for 0, 5, 60, or 180 min. Immunoprecipitation and western blot analysis were performed as in **a**. The *bottom panels* indicate the densitometric quantitation of phospho-EGFR levels relative to total EGFR levels. The data presented are the means ± SEM from four experiments

six-well plates, and then incubated with H_2-DCFDA (5 μM) for 30 min before adding 40 ng/ml SN38. The harvested cells were analyzed by means of a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.).

Isolation of membrane and cytoplasmic protein extracts

Cells were suspended in 200 μl ice-cold buffer (50 mM Tris-HCl, pH 7.4, 4 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, 10 μg/ml leupeptin, and 2 mM Na$_3$VO$_4$; pH 8.0) and clarified by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatants were centrifuged at 100,000 rpm for 60 min at 4°C. The resulting supernatant was used as the soluble cytoplasmic fraction. The pellets were

resuspended in RIPA buffer, and centrifuged at 10,000 rpm for 15 min at 4°C and the resulting supernatant was used as the soluble membrane fraction. After determining protein concentration, soluble proteins were subjected to immunoblot analysis.

Northern blot analysis

Cells were grown in 100-mm dishes to confluence. The cells were then serum-deprived for 24 h and exposed to 40 ng/ml SN38 with or without pretreatment with 1 μM gefitinib for a further 30 min. After 0, 3, 6, 12, and 24 h, total RNA was extracted using Sepasol-RNA I (Nacalai Tesque). Approximately 15 μg of each RNA was electrophoresed on 1.3% agarose/2.2 M formaldehyde denaturing gels, transferred to Hybond-XL membranes (Amersham, Little Chalfont, UK), and UV crosslinked (1200 J/m^2). Hybridization was performed using cDNA probes labeled using a random priming (Multiprime DNA Labeling System; Amersham) with [α-^{32}P]dCTP (Dupont-NEN, Boston, Mass.) in Rapid-hyb buffer (Amersham). Probes included a 590-bp *Kpn*I-*Eco*RI fragment of rat HB-EGF cDNA [7], a gift from Dr. M. Klagsbrun (Harvard Medical School, Boston, Mass.), a 750-bp *Bam*HI-*Eco*RI fragment for rat AR cDNA [7], a gift from Dr. G.D. Shipley (Oregon Health Sciences University, Portland, Ore.), a 0.45-kb *Eco*RI-*Eco*RI fragment of human IL-8 cDNA [17], a gift from Dr. N. Mukaida (Kanazawa University, Kanazawa, Japan), and a 1.1-kb *Xba*I-*Hin*dIII fragment of rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA [7],
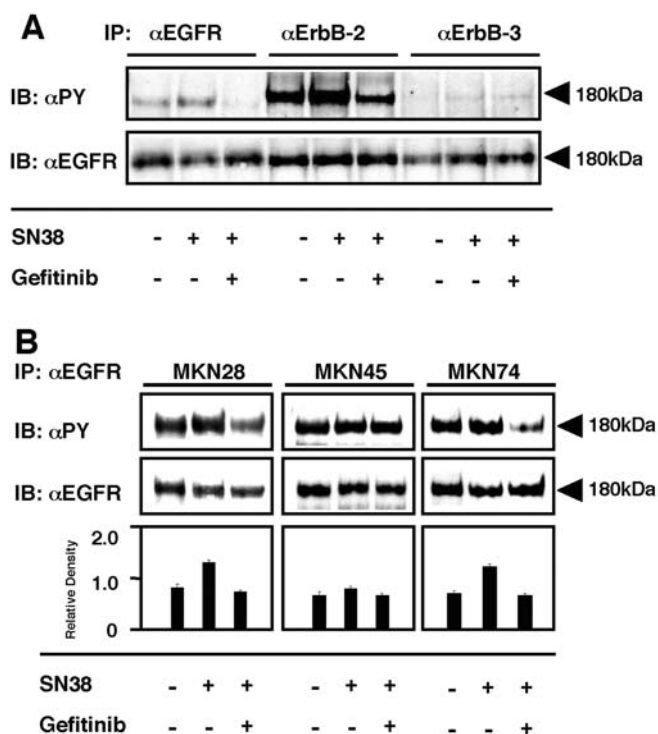
Fig. 2 a Gefitinib inhibits the SN38-induced tyrosine phosphorylation of EGFR, ErbB-2 in AGS cells. AGS cells were incubated with 40 ng/ml SN38 for 120 min after pretreatment with gefitinib for 30 min under the following conditions: *lane 1* untreated, *lane 2* SN38, *lane 3* SN38 with gefitinib (1 μM). Tyrosine phosphorylation of EGFR, ErbB-2, and ErbB-3 was determined by immunoprecipitation and Western blot analysis. The same filters were then stripped and reprobed with an anti-EGFR antibody (αEGFR). Each figure shows representative data from four similar experiments. **b** Gefitinib inhibits SN38-induced tyrosine phosphorylation of EGFR in gastric cancer cells. MKN28, MKN45, and MKN74 cells were incubated with SN38 for 120 min under the same conditions as in **a**. Immunoprecipitation and Western blot analysis were performed as in Fig. 1a. The *bottom panels* indicate the densitometric quantitation of phospho-EGFR levels relative to the total EGFR levels. The data presented are the means ± SEM from four experiments.

a gift from Dr. T. Nakamura (Osaka University Graduate School of Medicine, Osaka, Japan).

Nuclear extract preparation and electrophoretic mobility shift assay

Cells were pretreated with or without 1 μM gefitinib and then treated with 40 ng/ml SN38 for 0, 1, 3, or 8 h. Nuclear extracts from the cells were prepared using a modification of the procedure of Digman et al. [18]. The electrophoretic mobility shift assay (EMSA) for NF-κB and AP-1 was performed using a gel shift assay system (Promega Corporation, Madison, Wis.) according to the manufacturer's guidelines [17]. Double-stranded oligonucleotide probes for NF-κB (5′-AGTTGAGGGGAC TTTCCCAGGC-3′) and AP-1 (5′-CGCTTGATGAGT CAGCCGGAA-3′) were end-labeled with [γ-^{32}P]ATP. Each 10-μg sample of nuclear protein was incubated with a ^{32}P-labeled probe (5×10^4 cpm/reaction) and 0.5 μg/ml

poly(dI-dC) in 10 μl binding buffer (4% glycerol, 1 mM MgCl$_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl; pH 7.5) for 20 min at room temperature. Samples were loaded onto a 4% polyacrylamide gel (acrylamide/N,N′-methylene bisacrylamide, 30:1) with 0.5× Tris-borate EDTA buffer. After electrophoresis, the gels were dried and exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, N.Y.).

Measurement of TGF-α and IL-8 production by ELISA

Cells were seeded and grown to confluence in 24-well plates. The cells were treated with 40 ng/ml SN38 with or without pretreatment with 1 μM gefitinib for 2 h. After incubating the cells for 24 h, the concentrations of human TGF-α and IL-8 were determined using commercially available ELISA kits for TGF-α (Oncogene Research Products, Darmstadt, Germany) and IL-8 (Biosource International, Camarillo, Calif.) according to the manufacturers' guidelines.

Statistical analysis

Statistical comparisons were performed using one-way ANOVA with the Bonferroni/Dunn method. P values < 0.05 were considered significant.

Results

SN38 induces tyrosine phosphorylation of the EGF receptor in AGS cells

There have been only a few studies on the issue of whether genotoxic drugs are capable of inducing the tyrosine phosphorylation of EGFR, although these drugs are currently used in cancer treatment. Therefore, we first determined which materials were capable of activating EGFR in AGS cells. An MTT assay was used to determine what drug concentration to use. The lower concentration of each genotoxic drugs was based on IC$_{50}$values, while the higher concentration was set at ten times the lower concentration (Fig. 1a). As shown in Fig. 1a, a significant induction of tyrosine phosphorylation of EGFR by SN38 was apparent at 1 μM. To reveal how SN38 activated EGFR, the time-course for the activation was determined. As shown in Fig. 1b, the phosphorylation of EGFR was detected within 5 min and the intensity of the phosphorylated bands increased in a time-dependent manner up to 3 h in AGS cells; similar results were also obtained for MKN28 cells.

Gefitinib inhibits the SN38-induced tyrosine phosphorylation of EGFR in gastric cancer cells

It has been reported that high levels of EGFR, ErbB-2, and ErbB-3 are present in a number of gastric cancer cell
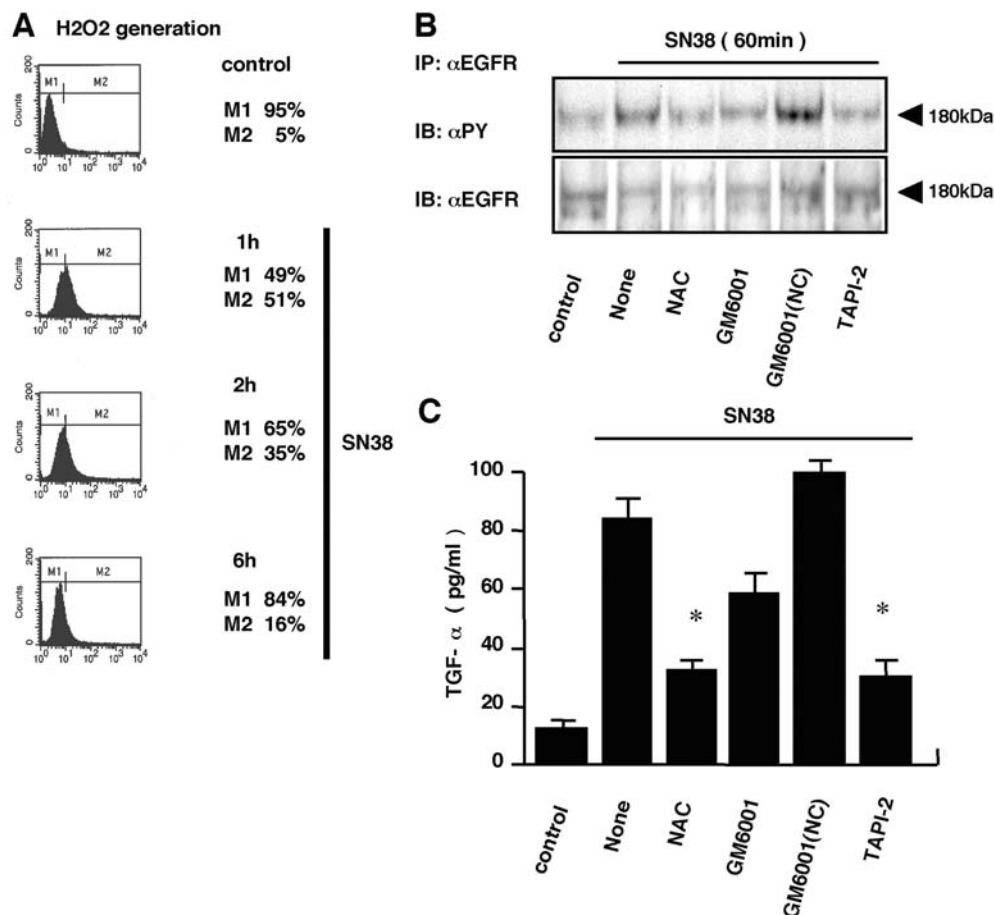
Fig. 3 a SN38-induced H$_2$O$_2$ generation. AGS cells were treated with 40 ng/ml SN38 for the indicated times before flow cytometric analysis. H$_2$-DCFDA was added 30 min prior to the measurements. The figure shows representative data from four similar experiments. **b** SN38-induced H$_2$O$_2$ and metalloproteinase-dependent EGFR transactivation. AGS cells were incubated with 40 ng/ml SN38 for 60 min after pretreatment with NAC or each inhibitor for 30 min under the following conditions: *lane 1* untreated, *lane 2* SN38, *lane 3* SN38 with NAC (10 m *M*), *lane 4* SN38 with GM6001 (10 *μM*), *lane 5* SN38 with GM6001 (*NC* negative control) (10 *μM*), *lane 6* SN38 with TAPI-2 (10 *μM*). Immunoprecipitation and Western blot analysis were performed as in Fig. 1a. Each figure shows representative data from four similar experiments. **c** SN38 stimulation resulted in the ectodomain shedding of EGFR ligands into the cell culture medium. AGS cells were incubated with 40 ng/ml SN38 after pretreatment with NAC or each inhibitor for 30 min under the following conditions: *lane 1* untreated, *lane 2* SN38, *lane 3* SN38 with NAC (10 m *M*), *lane 4* SN38 with GM6001 (10 *μM*), *lane 5* SN38 with GM6001 (*NC* negative control) (10 *μM*), *lane 6* SN38 with TAPI-2 (10 *μM*). After 24 h, the culture supernatant was examined for TGF-α production by ELISA. The data presented are the means ± SEM from six replicates. *$P < 0.05$ vs SN38 alone

lines [19]. Thus we next determined whether SN38 was able to activate these receptors in AGS cells. As shown in Fig. 2a, SN38 was able to induce not only phospho-EGFR, but also phospho-ErbB-2. Because gefitinib has been reported to affect signal transduction through different ErbB receptors by inducing the capture by EGFR of both ErbB-2 and ErbB-3 in the form of inactive dimers [20], we next examined whether gefitinib inhibited the SN38-induced phosphorylation of EGFR and ErbB-2. Gefitinib completely abrogated both of these phosphorylations (Fig. 2a). Similar result were obtained for MKN28 and MKN74, but not MKN45 (Fig. 2b). These results indicate that the SN38-induced activation of EGFR may depend on the cell type of the gastric cancer.

SN38 induces H$_2$O$_2$ generation and metalloproteinase-dependent EGFR transactivation

Although it has been reported that several genotoxic drugs generate H$_2$O$_2$ [6, 21], studies showing a relationship with the transactivation of EGFR are limited. We therefore examined whether SN38 induces H$_2$O$_2$, and results in activation of EGFR in AGS cells. The amount of intracellular H$_2$O$_2$ generated was determined using H$_2$-DCFDA. As shown in Fig. 3a, the content of H$_2$O$_2$ peaked within 1 h and then gradually decreased. Moreover, N-acetyl-L-cysteine (NAC), an antioxidant, inhibited SN38-induced EGFR activation (Fig. 3b). Because recent reports have indicated that H$_2$O$_2$ is able to induce the ectodomain shedding of EGFR ligands via the metalloproteinase-dependent pathway [22, 23], we next examined the effect of metalloproteinase inhibitors on SN38-induced EGFR activation. Figure 3b shows that a broad range metalloproteinase inhibitors, including GM6001, and the TNF-α-converting enzyme
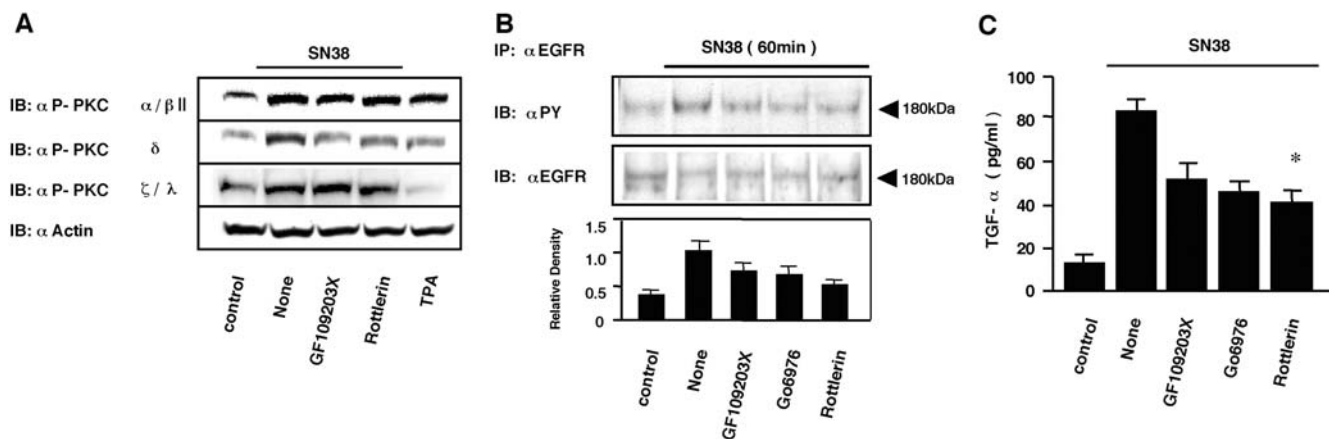
Fig. 4 a SN38-induced phosphorylation of PKC in AGS cells. AGS cells were incubated with 40 ng/ml SN38 for 60 min after pretreatment with each inhibitor for 30 min under the following conditions: *lane 1* untreated, *lane 2* SN38, *lane 3* SN38 with GF109203X (5 μM), *lane 4* SN38 with Rottlerin (5 μM), *lane 5* TPA (10 n M). Phosphorylation of PKC (α/βII, δ, ζ/λ) was determined by Western blot analysis. Equal loading of lanes was demonstrated by immunoblotting with anti-actin antibody after stripping anti-phospho-PKC antibody from the membrane. Each figure shows representative data from four similar experiments. **b** PKC mediates the transactivation of EGFR by SN38. AGS cells were incubated with 40 ng/ml SN38 for 60 min after pretreatment with each inhibitor for 30 min under the following conditions: *lane 1* untreated, *lane 2* SN38, *lane 3* SN38 with GF109203X (5 μM), *lane 4* SN38 with Go6976 (2.5 μM), *lane 5* SN38 with Rottlerin (5 μM). Immunoprecipitation and Western blot analysis were performed as in Fig. 1a. The *bottom panels* indicate the densitometric quantitation of phospho-EGFR levels relative to total EGFR levels. The data are presented as the means ± SEM from four experiments. **c** SN38 stimulation resulted in the ectodomain shedding of EGFR ligands into the cell culture medium. AGS cells were incubated with 40 ng/ml SN38 after pretreatment with each inhibitor for 30 min under the same conditions as in **b**. After 24 h, the culture supernatant was examined for TGF-α production using ELISA. The data presented are the means ± SEM from six replications. *$P \leq 0.05$ vs SN38 alone

(TACE) inhibitor TAPI-2 abrogated these effects. Furthermore, we evaluated whether SN38 stimulation led to the secretion of EGFR ligands into the cell culture medium. As shown in Fig. 3c, SN38 stimulation induced the secretion of TGF-α, whereas NAC and the metalloproteinase inhibitors partially abrogated these reactions. These results indicate that SN38-induced EGFR activation involves the generation of H_2O_2 and the metalloproteinase-dependent ectodomain shedding of EGFR ligands.

Activation of PKC mediates SN38-induced EGFR phosphorylation

A previous study has shown that tetradecanoyl-phorbol-13-acetate (TPA), an activator of protein kinase C (PKC), mediates the metalloproteinase-dependent cleavage of HB-EGF and its subsequent binding to EGFR [24]. To determine the other mechanism induced by SN38, we examined the issue of whether SN38

induces phosphorylation of protein-kinase C (PKC). As shown in Fig. 4a, SN38 induced phospho-classical PKC-α/βII, phospho-novel PKC-δ and phospho-atypical PKCζ/λ in the whole lysate, whereas phospho-novel PKC-θ was not induced (data not shown). In this experiment, we used two PKC inhibitors GF109203X (an α, β, γ, δ, ε inhibitor) and Rottlerin (a δ, θ inhibitor) as a negative control, and TPA (a classical and novel PKC activator) as a positive control. We next determined whether PKC is involved in SN38-induced EGFR phosphorylation. Three PKC inhibitors GF109203X, Go6976 (an α, β inhibitor), and Rottlerin partially inhibited SN38-induced EGFR phosphorylation (Fig. 4b). In addition, three PKC inhibitors partially inhibited the SN38-stimulated secretion of TGF-α into the cell culture medium (Fig. 4c). Therefore, these results suggest that the activation of different PKC isoforms mediates SN38-induced EGFR phosphorylation, at least in part.

H_2O_2 partially inhibits SN38-induced translocation to the membrane of phospho-PKC

To clarify the relationship between SN38 and H_2O_2 on the subcellular distribution of PKC, the antioxidant NAC (a precursor of glutathione) was used to investigate the translocation of phospho-PKC. The translocation of phospho-pan-PKC (α, βI, β, δ, ε, η) by SN38 with NAC from cytoplasm to the membrane increased in a time-dependent manner up to 180 min (Fig. 5a, c), whereas phopho-pan PKC induced by SN38 alone mainly increased in the cytoplasm (Fig. 5a, b). Therefore, these results suggest that H_2O_2 partially suppresses the SN38-induced translocation of phospho-PKC to the membrane at least up to 180 min.

SN38-induced EGFR ligands and inhibition by gefitinib

We next determined whether SN38 induced the production of EGFR ligands in AGS cells. Previous investigations have indicated that gastrointestinal epithelial
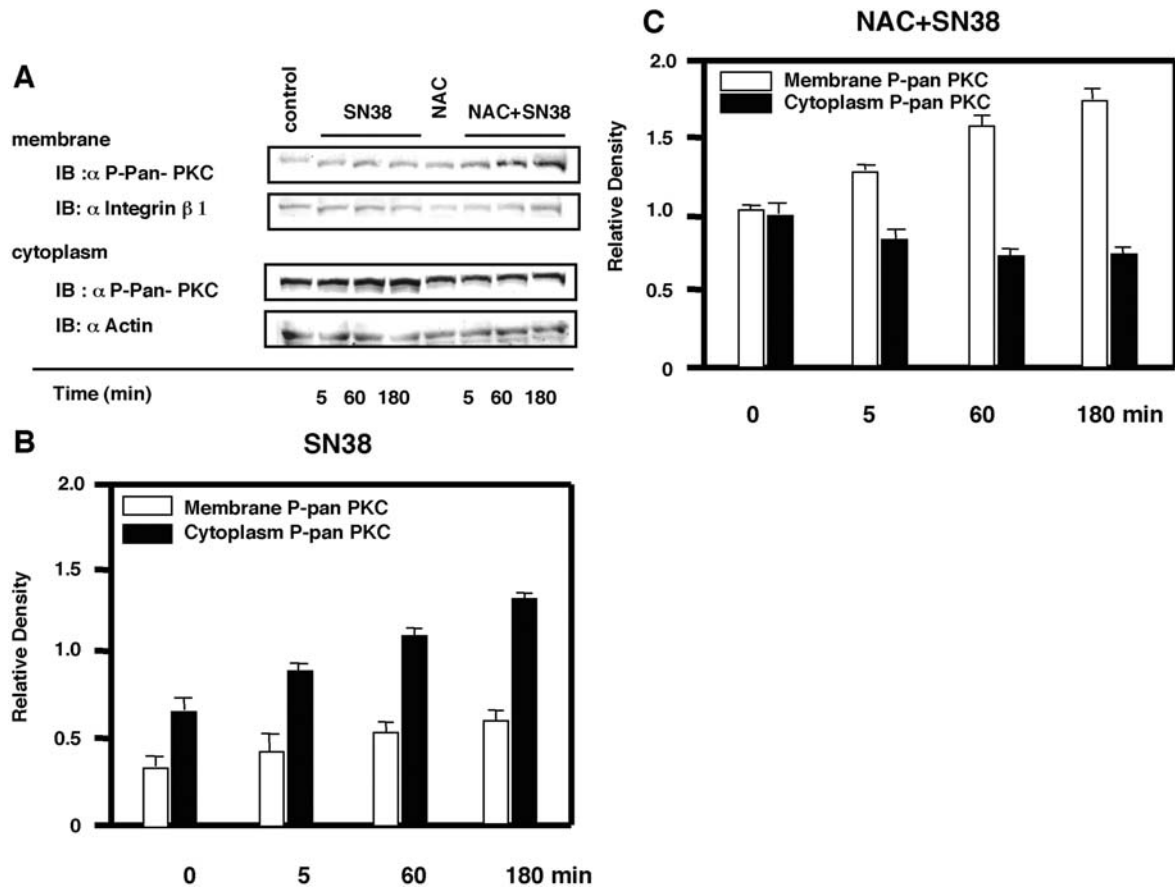
Fig. 5 a H_2O_2 partially inhibits SN38-induced translocation from cytoplasm to membrane of phospho-PKC. AGS cells were incubated with 40 ng/ml SN38 with or without NAC (10 m M) for 0, 5, 60, or 180 min. In some experiments, cells were preincubated with NAC (10 m M) for 30 min. The cells were then suspended in ice-cold lysis buffer and divided into a soluble cytoplasmic fraction and a cell membrane fraction by centrifugation. After determining the protein concentration, soluble proteins were subjected to immunoblot analysis. Immunoblots were probed with an anti-phospho-pan PKC antibody (α P-Pan PKC). The Filters were subsequently reprobed with an anti-Integrin $\beta1$ antibody (α Integrin $\beta1$; cell membrane) or an anti-actin antibody (α Actin; cytoplasm). **b, c** Densitometric quantitation of phospho-Pan-PKC levels (membrane or cytoplasm) relative to integrin $\beta1$ levels (membrane) or actin levels (cytoplasm) after treatment with SN38 (**b**) or SN38 with NAC (**c**) as shown in **a**. The data presented are the means ± SEM from four experiments

cells express HB-EGF, AR, and TGF-α [25]. The expression of HB-EGF and AR is regulated at the transcriptional level, while that of TGF-α is regulated mainly at the post-transcriptional level [25]. Thus, we determined the expression of HB-EGF and AR mRNA by Northern blotting and TGF-α protein by ELISA. Treatment with SN38 induced the expression of HB-EGF and AR transcripts in a time-dependent manner (Fig. 6a), and dramatically induced TGF-α secretion (Fig. 6c). Since previous work has indicated a critical role for EGFR in the expression of its ligands [7], we examined whether gefitinib inhibited the production of SN38-induced EGF-like growth factors. Gefitinib

inhibited HB-EGF and AR mRNA expression and the secretion of TGF-α protein (Fig. 6b, c).

SN38-induced IL-8 expression and its inhibition by gefitinib

It is well known that EGF is capable of inducing IL-8, which acts as an angiogenic factor in several solid tumors [26]. Thus we determined the effects of SN38 on the expression of IL-8 transcripts and protein. As shown in Fig. 7a, SN38 induced a biphasic expression of IL-8 mRNA and a 100% increase in IL-8 secretion into the culture medium. Gefitinib completely abrogated SN38-induced IL-8 mRNA expression and protein secretion (Fig. 7b, c).

SN38-induced NF-κB and AP-1 binding and its inhibition by gefitinib

The IL-8 promoter region contains three important *cis*-acting elements for IL-8 gene transcription, namely, binding sites for NF-κB, AP-1, and NF-IL-6. Transcription of the IL-8 gene requires the combination of either NF-κB and AP-1 or NF-κB and NF-IL-6 [27]. Therefore, we determined whether SN38 activates binding activity for NF-κB and AP-1 in AGS cells. Treatment with SN38 resulted in a persistent and
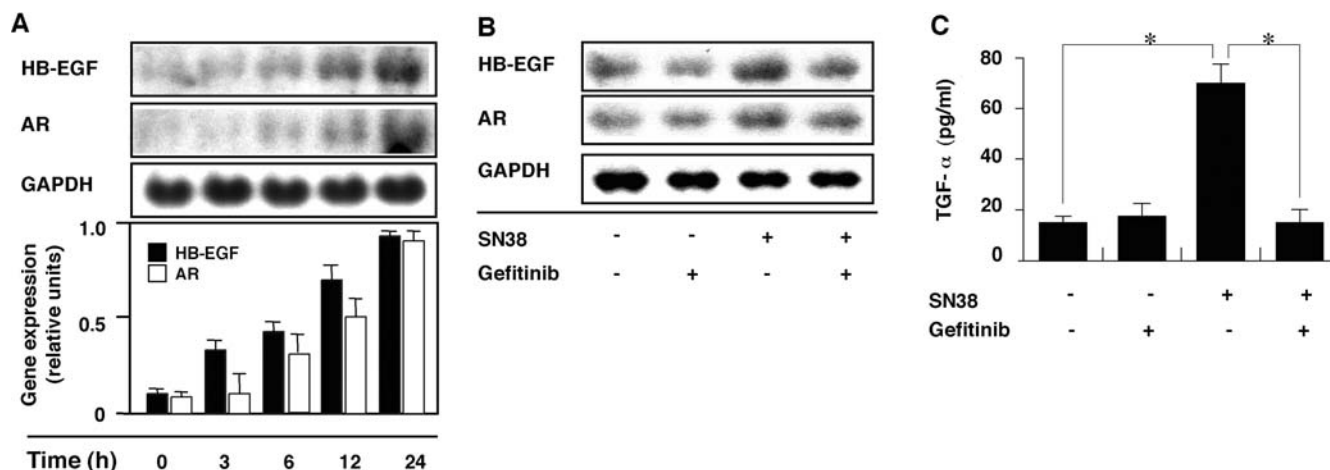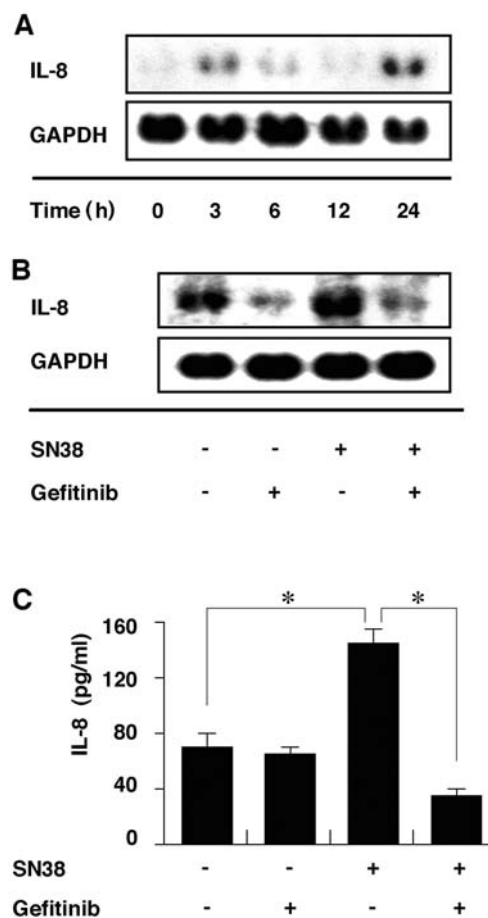
Fig. 6 a SN38-induced expression of HB-EGF and AR mRNA. AGS cells were incubated with 40 ng/ml SN38 for 0–24 h. Total RNA isolated from the cells was loaded and hybridized with cDNA probes for HB-EGF and AR. Blots were rehybridized with a GAPDH cDNA probe. The *bottom panel* indicates the densitometric quantitation of HB-EGF or AR mRNA levels relative to GAPDH mRNA levels. The data presented are the means ± SEM from four experiments. **b** Effect of gefitinib on SN38-induced expression of HB-EGF and AR mRNAs. AGS cells were incubated with SN38 for 6 h after pretreatment with or without gefitinib for 30 min under the following conditions: *lane 1* untreated, *lane 2* gefitinib (1 μM), *lane 3* SN38 (40 ng/ml), *lane 4* SN38 with gefitinib. Each autoradiograph is representative of four experiments. **c** Effect of gefitinib on SN38-induced TGF-α secretion from AGS cells. AGS cells were incubated with 40 ng/ml SN38 for 24 h under the same conditions as in **b**. After treatment, the culture supernatant was examined for TGF-α production using ELISA. The data presented are the means ± SEM from six replicates. *P < 0.05 vs SN38 alone

time-dependent activation of NF-κB up to 8 h, whereas the binding activity of AP-1 reached a maximal level within 1 h (Fig. 8a, b). Gefitinib completely abrogated the activation of NF-κB and AP-1. A suggested mechanism for the action of gefitinib is presented in Fig. 9.

Discussion

The present study showed that SN38 (the active metabolite of CPT-11) is capable of inducing the tyrosine phosphorylation of EGFR and the expression of EGF-like growth factors, including HB-EGF, AR, and TGF-α in gastric cancer cells. The study also showed that SN38 activates the EGF/EGFR autocrine loop and induces IL-8. As gefitinib abrogated all these reactions, its combination with CPT-11 may be a potential therapeutic approach in the treatment of certain gastric cancers.

A variety of carcinoma cells including gastric cancers express EGFR and its ligands at high levels, and these have been implicated in the development and progression of tumors [28]. The activation of EGFR is not only critical for cell proliferation but is also contributes to other processes crucial to cancer progression, including angiogenesis, metastatic spread, and inhibition of


Fig. 7 a SN38-induced expression of IL-8 mRNA. AGS cells were incubated with 40 ng/ml SN38 for 0–24 h. Total RNA isolated from the cells was loaded and hybridized with a cDNA probe for IL-8. Blots were rehybridized with a GAPDH cDNA probe. Each autoradiograph is representative of four experiments. **b** Effect of gefitinib on SN38-induced expression of IL-8 mRNA. AGS cells were incubated with SN38 for 3 h under the same conditions as in Fig. 6b. Each autoradiograph is representative of four experiments. **c** Effect of gefitinib on SN38-induced IL-8 secretion from AGS cells. AGS cells were incubated with 40 ng/ml SN38 for 24 h under the same conditions as in Fig. 6b. After treatment, the culture supernatant was examined for IL-8 production using ELISA. The data presented are the means ± SEM from six replications. *P < 0.05 vs SN38 alone
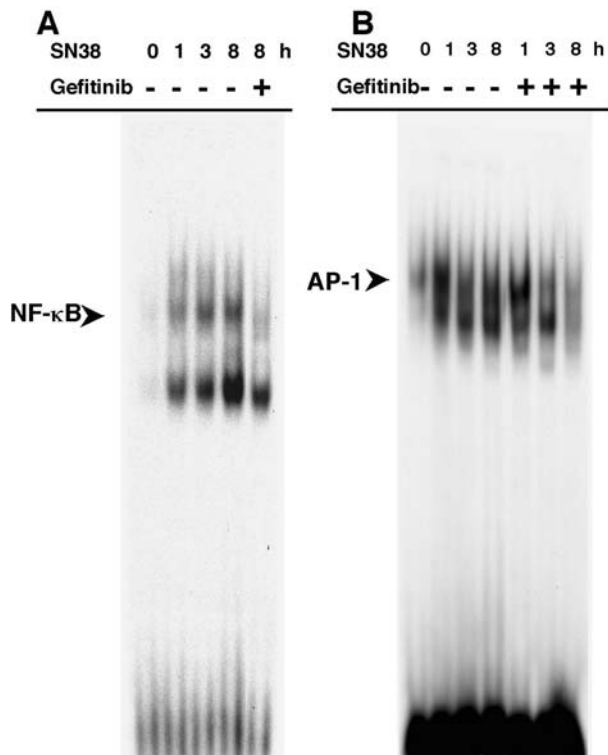
Fig. 8 a Effect of SN38 on the transcriptional activity of NF-κB and AP-1 as assessed by EMSA. AGS cells were incubated with 40 ng/ml SN38 with or without 1 μM gefitinib for 0–8 h. Nuclear proteins extracted from the cells were incubated with radiolabeled oligonucleotide probes for NF-κB (**a**) and AP-1 (**b**). Each autoradiograph is representative of four experiments
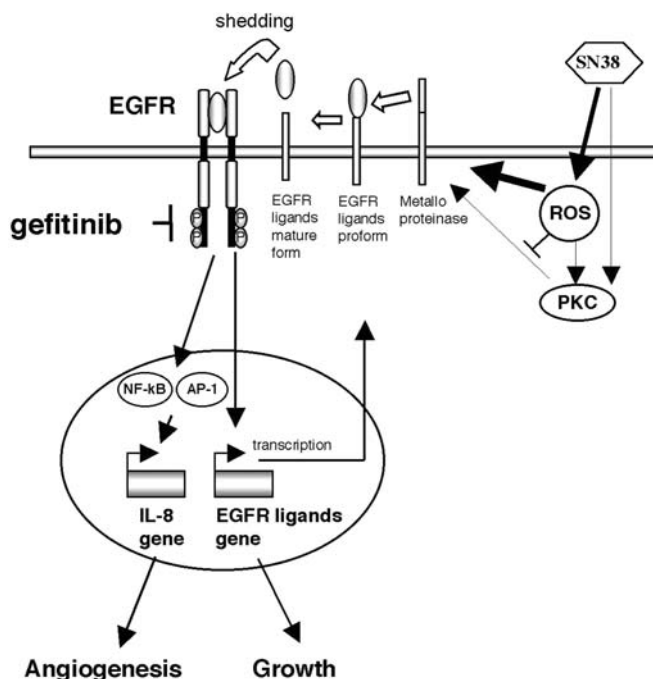
Fig. 9 Gefitinib inhibits SN38-triggered EGF signals and IL-8 production, resulting from ROS generation and PKC activation, followed by metalloproteinase activation and sequential ectodomain shedding of EGFR ligands

apoptosis [10]. Thus, the EGF/EGFR system could play an important role in the cell survival response to block apoptotic signals in cancer cells that have been exposed to cytotoxic damage. Our results suggest that the unexpected activation of the EGF/EGFR system by SN38 could contribute to resistance of certain types of cancers to CPT-11. In addition, gefitinib inhibited not only the tyrosine phosphorylation of EGFR but also the induction of EGF-like growth factors by SN38. These results suggest that EGFR plays a primary role in the induction of EGF-like growth factors by SN38.

A previous report has shown that EGFR activation is rapidly induced by EGF and then rapidly decreases, but remains increased in the presence of H_2O_2 during all time intervals examined [8, 29]. This suggests that several mechanisms related to H_2O_2-induced EGFR transactivation may be operative. For example, in the rapid response, H_2O_2 may interact directly with the receptor and cell membrane, leading to changes in receptor conformation and membrane structure, resulting in the activation of the receptor [8]. In addition, the persistent activation of EGFR may depend on failing to stimulate c-Cbl-mediated downregulation [29]. The present results indicate some novel aspects in which the rapid and persistent activation of EGFR by SN38 leads not only to the generation of H_2O_2 but also to the activation of PKC, followed by the metalloproteinase-dependent ectodomain shedding of EGFR ligands.

A previous study has indicated that diverse PKC isoforms, including α, βI, and γ of classical-PKC, δ and ε of novel-PKC, and ζ of atypical-PKC, are activated in response to H_2O_2 [30]. The reaction by SN38 is consistent with this finding. However, SN38 with NAC (an antioxidant) also activated pan-PKC, and permitted translocation of PKCs from the cytoplasm to the membrane (Fig. 5). Thus, SN38 may activate diverse PKCs by a direct or indirect mechanism via H_2O_2, resulting in the activation of metalloproteinase. In the case of PKC isoforms, PKC δ is known to be required for the apoptotic response of cells to oxidative stress [31, 32] whereas the overexpression of PKC α or β1 may play a role in the multidrug resistance of gastrointestinal cancer cells [33, 34]. Thus, our findings suggest that SN38 may induce proapoptotic signals through the activation of PKC δ and also induce the production of survival signals through the activation of EGFR by H_2O_2 or certain PKCs.

The present study demonstrated that SN38 is able to upregulate the expression of IL-8 in AGS cells. IL-8 is a CXC chemokine which is known to play a role in the recruitment of leukocytes and in the initiation and perpetuation of the inflammatory process [27]. In addition, along with vascular endothelial growth factor and basic fibroblast growth factor, IL-8 also appears to be an angiogenic factor, and may be implicated in tumor progression [10]. Recent studies have shown that high levels of expression of IL-8 in gastric cancers are associated with advanced disease and a poor prognosis [35]. The results of our study suggest that

CPT-11 may have an angiogenic effect on certain tumors through the induction of IL-8.

A variety of cytokines, hormones, and growth factors are able to induce IL-8 expression in epithelial cells [17, 27]. Previous studies have shown that EGFR-mediated signals contribute to the expression of IL-8, and IL-8 may be involved, at least in part, in EGF/EGFR-induced cancer progression [10]. It has been shown that the expression of IL-8 is regulated mainly at the transcriptional level and that NF-κB, AP-1, and NF-IL-6 contribute to the transcription of the IL-8 gene [27]. A recent study has indicated that SN38 activates NF-κB through mobilization and stimulation of the classical Iκ kinase (IKK) complex [36]. However, the issue of SN38-activated upstream signals leading to the IKK complex formation has not been investigated. The present study demonstrated that gefitinib inhibits SN38-induced IL-8 expression, indicating that EGFR mediates this reaction. Moreover, SN38 induced binding activities in both NF-κB and AP-1, critical transcription factors for the expression of IL-8, and this reaction was inhibited by gefitinib. Collectively, these findings suggest that SN38 activates the EGF/EGFR autocrine loop, which mediates the expression of IL-8 through the activation of NF-κB and AP-1.

Although the topoisomerase inhibitor CPT-11 is used clinically in the treatment of several solid tumors including gastric cancer [3, 4], the mechanisms of the differential sensitivity to CPT-11 remain unknown. The present study suggests that the SN38-induced activation of the EGF/EGFR autocrine loop and IL-8 expression may be involved in the resistance of certain gastric cancer cells to CPT-11. Since EGFR tyrosine kinase inhibition by gefitinib abrogates these SN38-induced reactions, it is possible that a combination of CPT-11 with gefitinib may be a potential therapeutic approach to the treatment of certain gastric cancers. Further studies are required to determine whether gefitinib reverses the resistance to CPT-11 in gastric cancer.

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