Cancer Chemother Pharmacol (2005) 55: 393–403
DOI 10.1007/s00280-004-0904-0

ORIGINAL ARTICLE

**Osamu Kishida · Yoshiji Miyazaki · Yoko Murayama
Miyuki Ogasa · Tamana Miyazaki
Takahiro Yamamoto · Kenji Watabe
Shusaku Tsutsui · Tatsuya Kiyohara
Iichiro Shimomura · Yasuhisa Shinomura**

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

Received: 10 January 2004 / Accepted: 4 June 2004 / Published online: 5 October 2004

Abstract The epidermal growth factor receptor (EGFR) and its ligands are involved in tumor growth, metastasis, angiogenesis, and resistance to chemotherapy. The findings reported here demonstrate that SN38 (the active metabolite of CPT-11) induces the tyrosine phosphorylation of EGFR within 5 min, followed by the induction of transcripts and/or proteins of the heparin-binding EGF-like growth factor, amphiregulin, transforming growth factor-α, and interlukin-8 (IL-8) in AGS gastric cancer cells. 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 · Epidermal growth factor · Epidermal growth factor receptor · SN38 · Interleukin-8

“Iressa” is a trademark of the AstraZeneca group of companies.

O. Kishida (✉) · Y. Miyazaki · Y. Murayama · M. Ogasa
T. Miyazaki · T. Yamamoto · K. Watabe · S. Tsutsui
T. Kiyohara · I. Shimomura · Y. Shinomura
Department of Internal Medicine and Molecular Science,
Graduate School of Medicine, Osaka University,
2-2 Yamadaoka, Suita, Osaka, 565-0871, Japan
E-mail: osamuk@imed2.med.osaka-u.ac.jp
Tel.: +81-6-68793732
Fax: +81-6-68793739

Abbreviation AP-1: Activator protein-1 · AR: Amphiregulin · CPT: Camptothecin · EGF: Epidermal growth factor · EGFR: EGF receptor · ELISA: Enzyme-linked 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

Introduction

Camptothecin (CPT), a DNA topoisomerase inhibitor, blocks the 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 clinically used in 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]. Above normal levels of ROS 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 recently found that oxidative stress, paradoxically, induces the rapid tyrosine phosphorylation of the epidermal growth factor receptor (EGFR), followed by a marked induction of EGF-like growth factors in a gastric epi-

thelial 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 transactivates 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 participate in the regulation of a variety of cell functions including cell growth, migration, survival, and angiogenesis [10], the activation of the 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 antitumor activity with respect to ionizing radiation and genotoxic drugs as the result of 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 the tyrosine phosphorylation of EGFR, the expression of EGF-like ligands, the activation of activator protein-1 (AP-1) and nuclear factor-kappaB (NF-κB), and the 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.) except *N*-acetyl-L-cysteine (NAC) which were purchased from Sigma-Aldrich (St Louis, Mo.). Genotoxic drugs, except SN38, were also purchased from Sigma-Aldrich. The ROS-sensitive fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, diacetoxymethyl-ester (H_2-DCFDA) was purchased from Sigma-Aldrich. 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, α/βII, δ, θ, ζ/λ- protein kinase C (PKC) antibody, mouse anti-phosphotyrosine monoclonal antibody (p-thy-100), HRP-linked anti-mouse IgG and HRP-linked antirabbit IgG were purchased from Cell Signaling Technology (Beverly, Mass.). The mouse anti-Integrin βI 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 (BioWhittaker, Walkersville, Md.), and incubated with 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 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 1% NP-40, 0.5% deoxycholate-Na, 0.1% SDS, 1 m*M* phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, 10 μg/ml leupeptin, and 2 m*M* Na_3VO_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 blot was placed in the appropriate HRP-conjugated secondary antibody for 60 min. Proteins were then detected using an enhanced chemiluminescence system (ECL, Amersham, Little Chalfont, UK). In some experiments, the membrane was then stripped and reprobed with the appropriate antibody as an internal control to verify equivalent protein loadings

Evaluation of intracellular ROS

Intracellular ROS levels were estimated using an oxidant-sensitive H_2-DCFDA probe. Cells were grown in 6-well multiwell 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_3VO_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 an 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 by Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan). 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. Gray 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], a gift from Dr. T. Nakamura (Osaka University Graduate School of Medicine, Osaka, Japan).

Nuclear extract preparation and EMSA

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 Digman's procedure [18]. An electrophoretic mobility shift assay (EMSA) for NF-κB and AP-1was 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′-AGTTGAGGGGACTTTCCCAGGC-3′) and AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-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 a 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 to be significant.

Results

SN38 induces tyrosine phosphorylation of the EGF receptor in AGS cells

In only a few studies has the issue of whether genotoxic drugs are capable of inducing the tyrosine phosphorylation of EGFR been investigated, 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 drug 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,

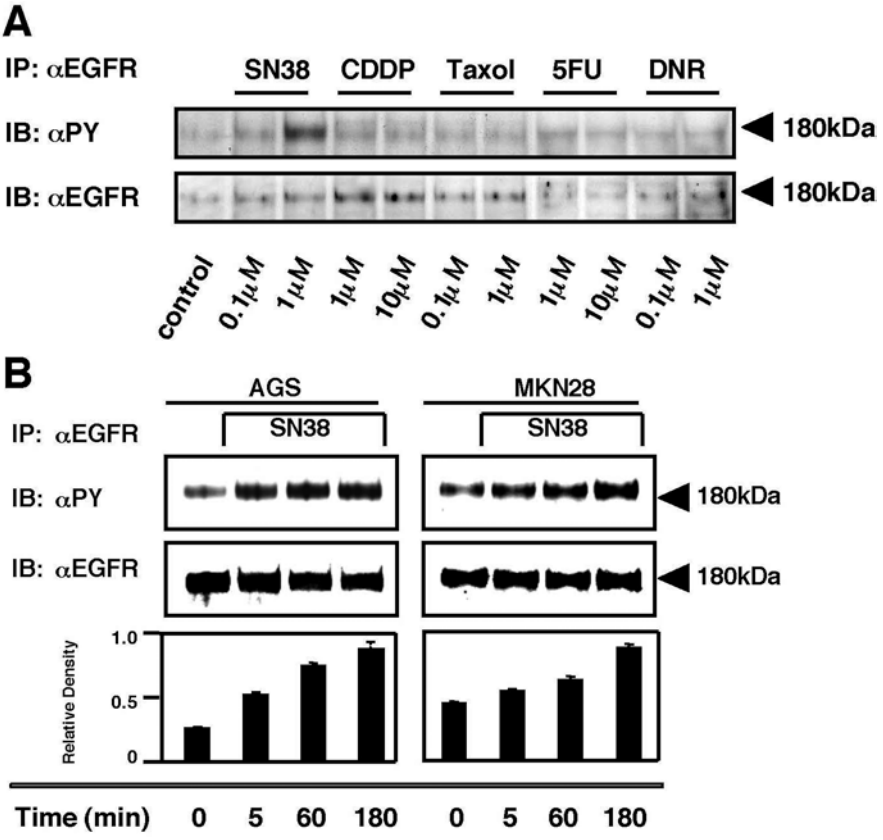
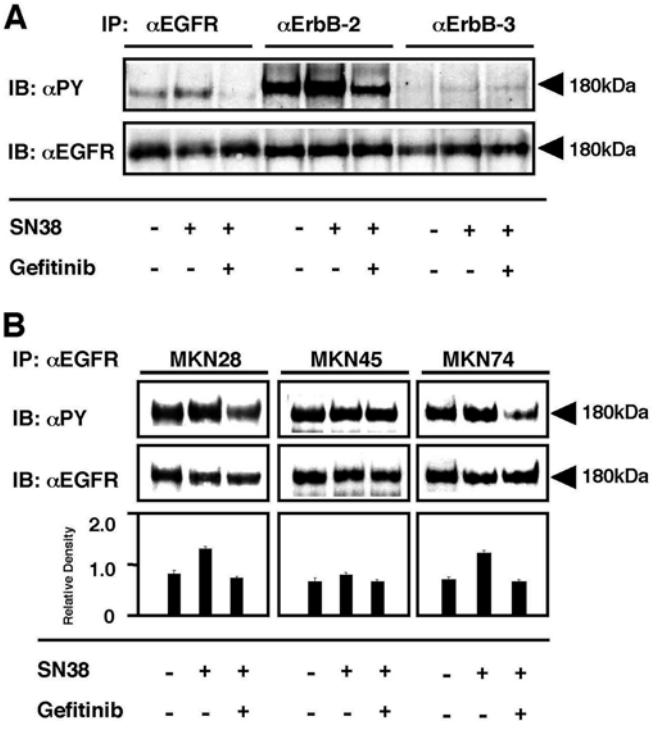


Fig. 1 **a** SN38-induced tyrosine phosphorylation of EGF receptor in AGS cells. AGS cells were incubated with SN38, cisplatin (*CDDP*), paclitaxel (*Taxol*), 5-fluorouracil (*5-FU*), and daunorubicin (*DNR*) for 120 min at the indicated concentrations. The cells were then lysed, and immunoprecipitation was performed using the anti-EGF receptor antibody. The precipitates were separated by SDS-PAGE and transferred to membranes. Immunoblots were probed with an antiphosphotyrosine 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 or MKN28 cells. Each of the cell types was incubated with 40 ng/ml SN38 for 0, 5, 60, or 180 min. Immunoprecipitation and Western blot analysis were performed as in **a**. *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

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, and the same results were also obtained for MKN28 cells.

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

Since it has been reported that high levels of EGFR, ErbB-2, and ErbB-3 are present in a number of gastric cancer cell lines [19], 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 EGFR to capture both ErbB-2 and ErbB-3 in the form of inactive dimers [20], we next examined whether



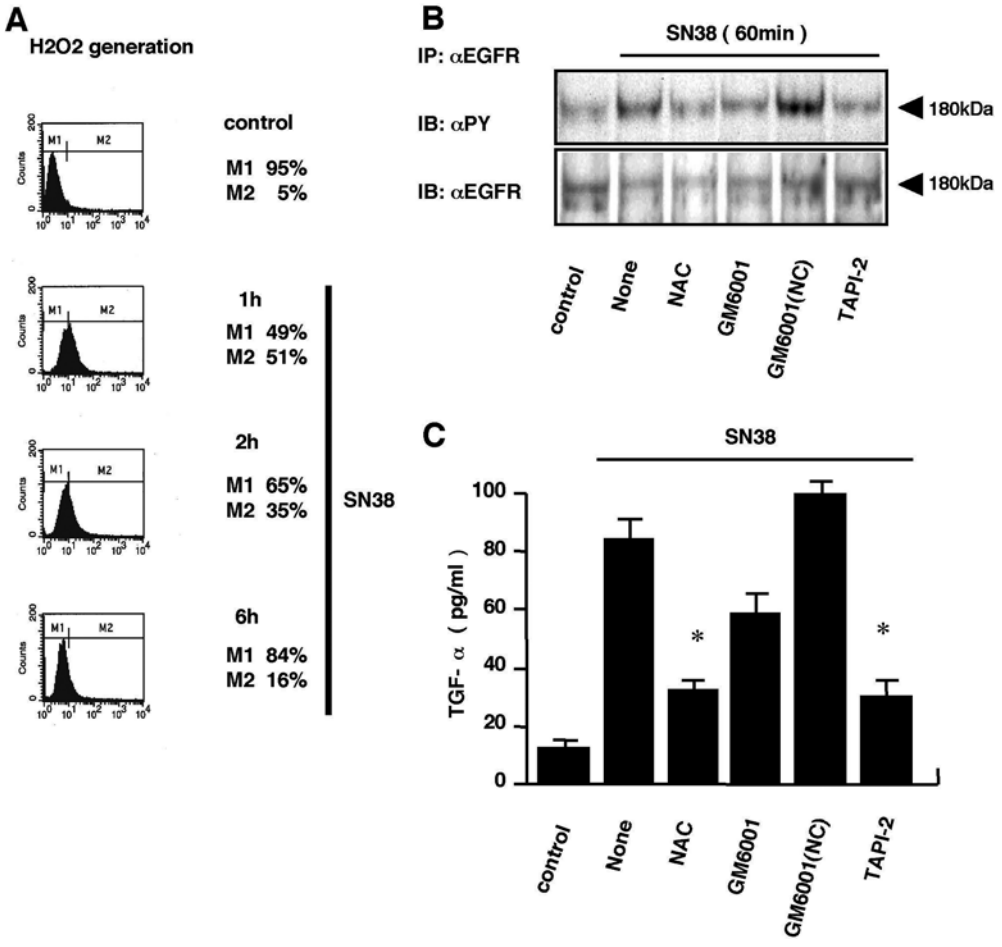


Fig. 3 **a** SN38-induced H_2O_2 generation. AGS cells were treated with 40 ng/ml SN38 for the indicated times before flow cytometric analysis. H_2-DCFDA was added 30 min prior to the measurements. The figure shows representative data from four similar experiments. **b** SN38-induced H_2O_2 and metalloproteinase-dependent 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 described 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, TGF-α was determined in the culture supernatant by ELISA. The data presented are the means ± SEM from six replications. $^*P < 0.05$ vs SN38 alone

◀

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 were 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 described in Fig. 1a. *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

gefitinib inhibits the SN38-induced phosphorylation of EGFR and ErbB-2. The findings indicated that 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_2O_2 generation and metalloproteinase-dependent EGFR transactivation

Although it has been reported that several genotoxic drugs generate H_2O_2 [6, 21], there are few reports of a

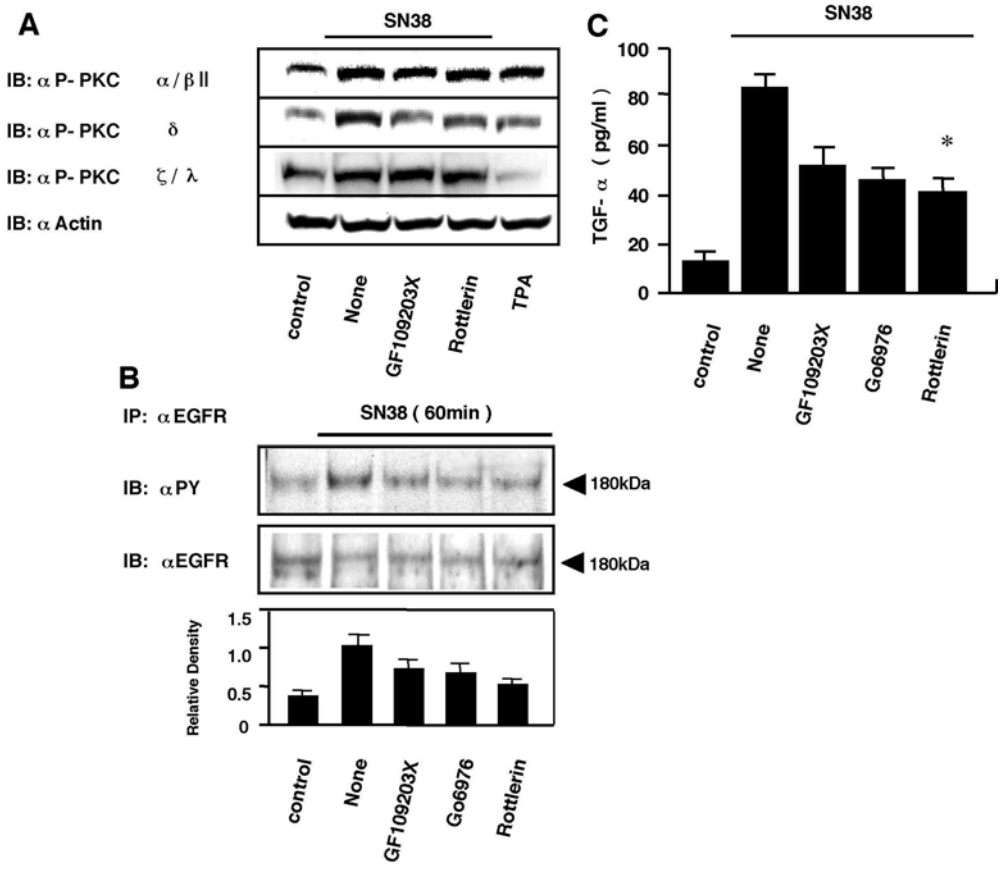


Fig. 4 **a** SN38-induced phosphorylation of PKC in AGS cells. AGS cells were incubated with 40 ng/ml SN38 for 60 min after pretreatment with each inhibitor for 30 min under the following conditions: *lane 1* untreated, *lane 2* SN38, *lane 3* SN38 with GF109203X (5 μM), *lane 4* SN38 with Rottlerin (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 described in Fig. 1a. *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. **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, TGF-α was determined in the culture supernatant using ELISA. The data presented are the means ± SEM from six replications. *$P < 0.05$ vs SN38 alone

relationship with the transactivation of EGFR. We therefore examined whether SN38 induces H_2O_2, and results in activation of EGFR in AGS cells. The amount of intracellular H_2O_2 generated was determined using H_2-DCFDA. As shown in Fig. 3a, the content of H_2O_2 peaked within 1 h and then gradually decreased. Moreover, NAC, an antioxidant, inhibited SN38-induced EGFR activation (Fig. 3b). Because recent reports have indicated that H_2O_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 of metalloproteinase inhibitors, including GM6001, and the TNF-α converting enzyme (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 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 the phosphorylation of

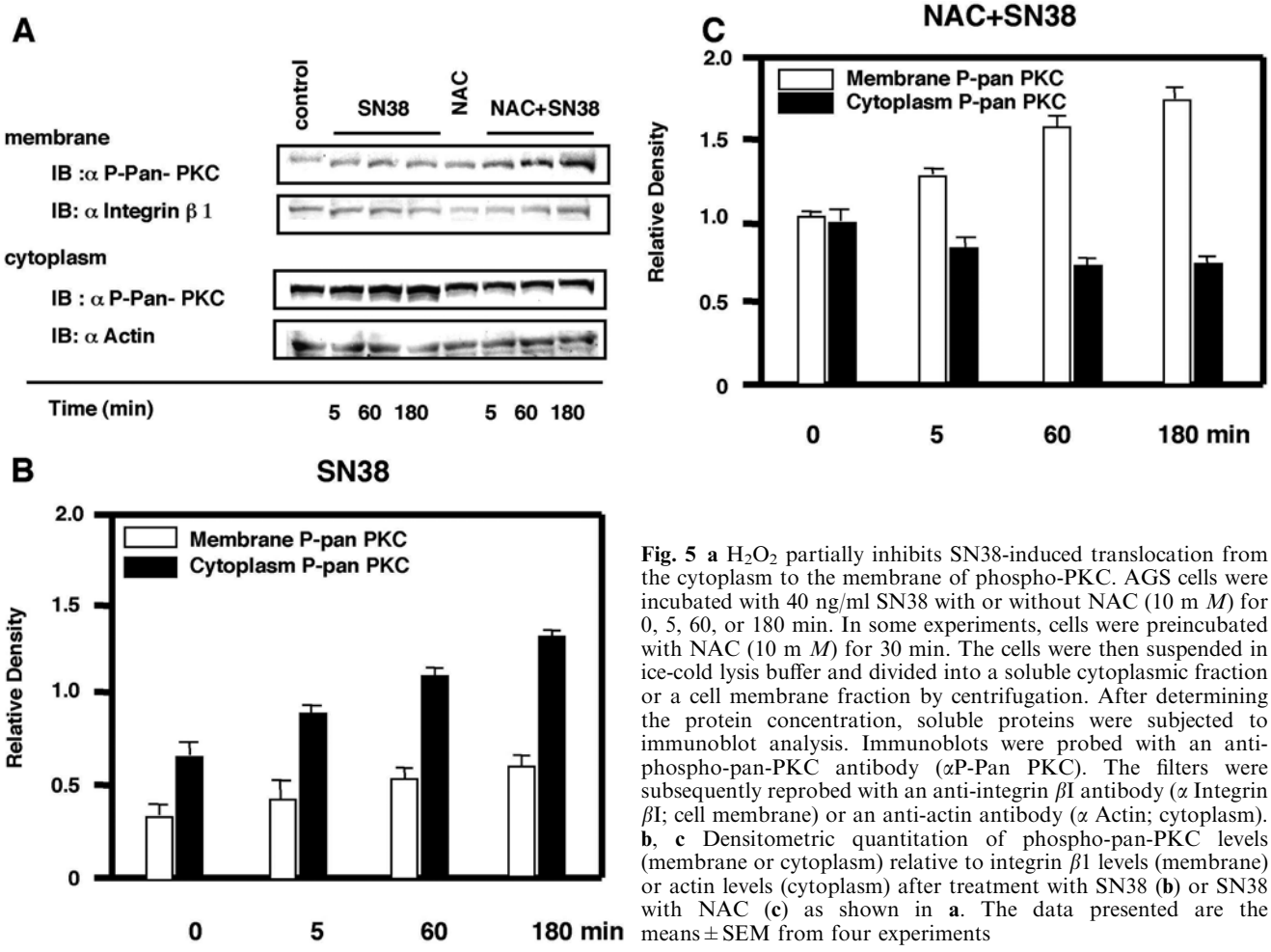


Fig. 5 **a** H_2O_2 partially inhibits SN38-induced translocation from the cytoplasm to the membrane of phospho-PKC. AGS cells were incubated with 40 ng/ml SN38 with or without NAC (10 m *M*) for 0, 5, 60, or 180 min. In some experiments, cells were preincubated with NAC (10 m *M*) for 30 min. The cells were then suspended in ice-cold lysis buffer and divided into a soluble cytoplasmic fraction or 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 (αP-Pan PKC). The filters were subsequently reprobed with an anti-integrin βI antibody (α Integrin βI; 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 β1 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

PKC. As shown in Fig. 4a, SN38 induced phospho-classical PKC-α/βII, phospho-novel PKC-δ, and phospho-atypical PKC-ζ/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 negative controls, 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 relationships between SN38 and H_2O_2 and the subcellular distribution of PKC, antioxidants NAC (a precursor of glutathione) were used to investigate the translocation of phospho-PKC. The translocation of phospho-pan-PKC (α, βI, βII, δ, ϵ, η) by SN38 with NAC from the cytoplasm to the membrane increased in a time-dependent manner up to 180 min (Fig. 5a,c), whereas phospho-pan-PKC induced by SN38 alone increased mainly 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 indicate that the gastrointestinal epithelial 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

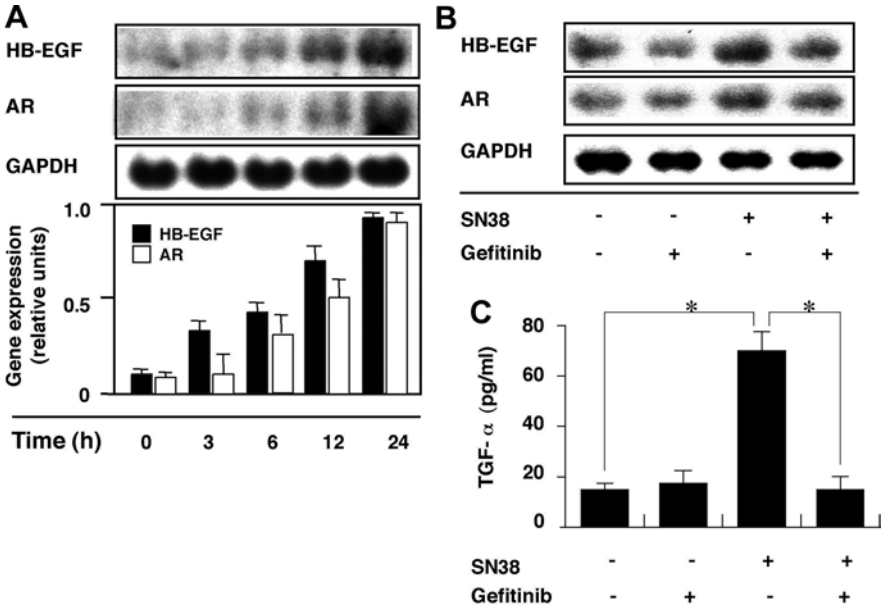


Fig. 6 **a** SN38-induced expression of HB-EGF and AR mRNA. AGS cells were incubated with 40 ng/ml SN38 for 0–24 h. Total RNA isolated from the cells was loaded and hybridized with cDNA probes for HB-EGF and AR. Blots were rehybridized with a GAPDH cDNA probe. *Bottom panels* indicate the densitometric 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, TGF-α was determined in the culture supernatant using ELISA. The data presented are the means ± SEM from six replications. $*P < 0.05$ vs SN38 alone

transcripts in a time-dependent manner (Fig. 6a), and dramatically induced TGF-α secretion (Fig. 6c). Since the previous work 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. On the other hand, 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, NF-κB binding sites, AP-1 binding sites, and NF-IL-6 binding sites. Transcription of the IL-8 gene requires either the combination of NF-κB and AP-1 or NF-κB and NF-IL-6 [27]. Therefore, we determined whether SN38 activates NF-κB and AP-1 binding activity in AGS cells. Treatment with SN38 resulted in a persistent and time-dependent activation of NF-κB up to 8 h, whereas the binding activities of AP-1 reached a maximal level within 1 h (Fig. 8a,b). On the other hand, gefitinib completely abrogated the activation of NF-κB and AP-1.

Discussion

The present study show 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 findings also show that SN38 activates the EGF/EGFR autocrine loop and induces IL-8. As gefitinib abrogates all these reactions, a combination of it 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, which have been implicated in the development and progression of tumors [28]. The activation of EGFR is not only critical for cell proliferation but also contributes to other processes that are crucial to cancer progression, including angiogenesis, metastatic spread, and the inhibition of apoptosis [10]. Thus, the EGF/EGFR system could play

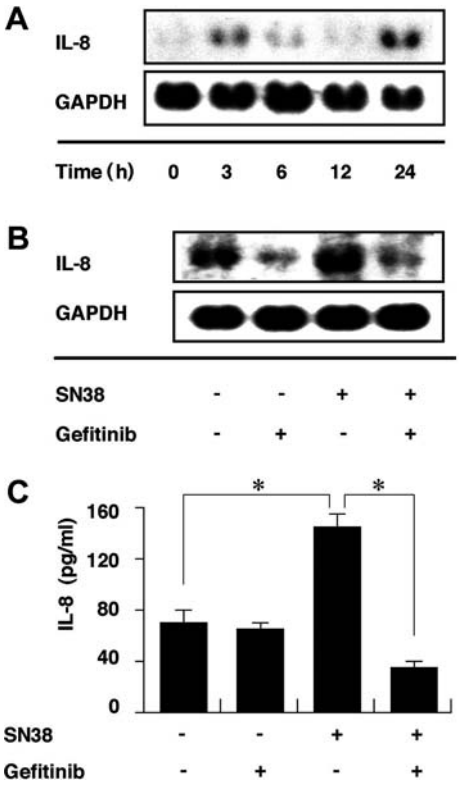


Fig. 7 **a** SN38-induced expression of IL-8 mRNA. AGS cells were incubated with 40 ng/ml SN38 for 0–24 h. Total RNA isolated from the cells was loaded and hybridized with a cDNA probe for IL-8. Blots were rehybridized with a GAPDH cDNA probe. Each autoradiograph is representative of four experiments. **b** Effect of gefitinib on SN38-induced expression of IL-8 mRNAs. AGS cells were incubated with SN38 for 3 h under the same conditions as described 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 described in Fig. 6b. After treatment, IL-8 was determined in the culture supernatant using ELISA. The data presented are the means ± SEM from six replications. $*P < 0.05$ vs SN38 alone

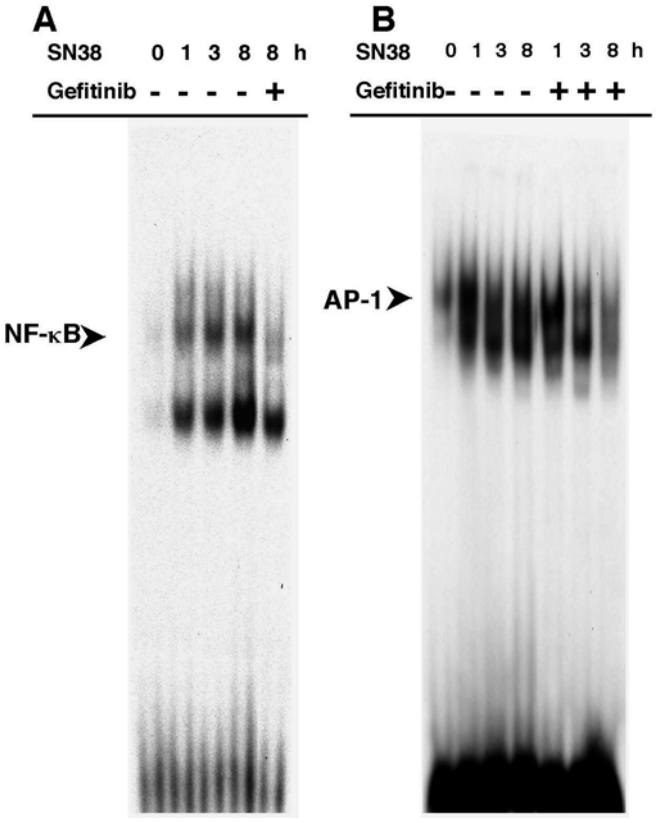


Fig. 8 **a** Effect of SN38 on the transcriptional activity of NF-κB and AP-1 as assessed by EMSA. AGS cells were incubated with 40 ng/ml SN38 with or without 1 μM gefitinib for 0–8 h. Nuclear proteins extracted from the cells were incubated with radiolabeled oligonucleotide probes for NF-κB (**a**) and AP-1 (**b**). Each autoradiograph is representative of four experiments

an important role in the cell survival response to block apoptotic signals in cancer cells that have been exposed to cytotoxic damage. Our findings suggest that the unexpected activation of the EGF/EGFR system by SN38 could contribute to resistance to CPT-11 by certain types of cancers. 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 study has shown that EGFR activation by EGF is rapidly induced and then rapidly decreased, but that this reaction by H_2O_2 remains increased 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 the 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 lead, 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 to SN38 is consistent with this report. However, SN38 with NAC (an antioxidant) also activated pan-PKC, and permitted their translocation 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 βI 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

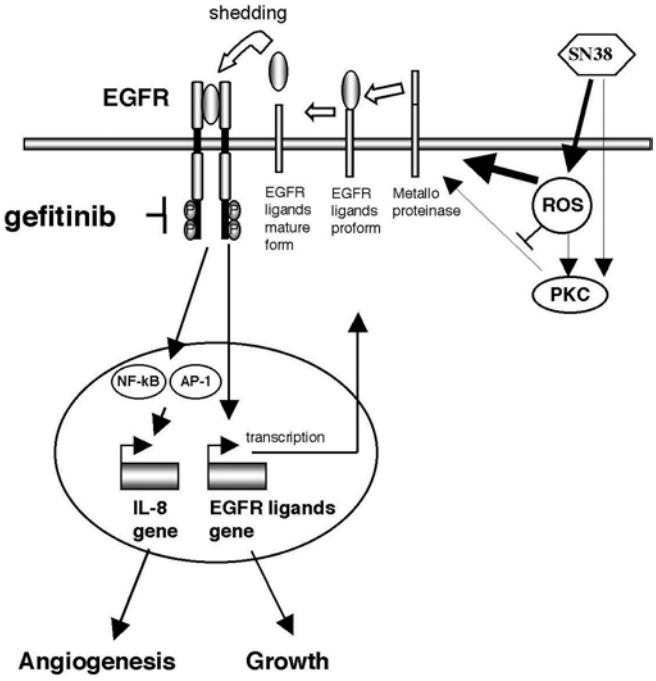


Fig. 9 Gefitinib inhibits SN38-triggered EGF signals and IL-8 production, resulting from ROS generation and PKC activation, followed by metalloproteinase activation and sequential ectodomain shedding of EGFR ligands

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 can 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 that 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 inhibited SN38-induced IL-8 expression, indicating that EGFR mediates this reaction. A suggested mechanism for the action of gefitinib is presented in Fig. 9. 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 clinically used in the treatment of several solid tumors including gastric cancers [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.

Acknowledgements This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Pommier Y, Pourquier P, Fan Y, Strumberg D (1998) Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. Biochim Biophys Acta 1400:83–105
2. Kunimoto T, Nitta K, Tanaka T, Uehara N, Baba H, Takeuchi M, Yokokura T, Sawada S, Miyasaka T, Mutai M (1987) Antitumor activity of 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxy-camptothecin, a novel water-soluble derivative of camptothecin, against murine tumors. Cancer Res 47:5944–5947
3. Bleiberg H (1999) CPT-11 in gastrointestinal cancer. Eur J Cancer 35:371–379
4. Ajani JA, Baker J, Pisters PW, Ho L, Mansfield PF, Feig BW, Charnsangavej C (2001) CPT-11 plus cisplatin in patients with advanced, untreated gastric or gastroesophagus junction carcinoma. Cancer 94:641–646
5. Motwani M, Sirotnak FM, She Y, Commes T, Schwartz K (2002) Drg1, a novel target for modulating sensitivity to CPT-11 in colon cancer cells. Cancer Res 62:3950–3955
6. Hiraoka W, Vazquez N, Nieves-Neira W, Chanock SJ, Pommier Y (1998) Role of oxygen radicals generated by NADPH oxidase in apoptosis induced in human leukemia cells. J Clin Invest 102:1961–1968
7. Miyazaki Y, Hiraoka S, Tsutsui S, Kitamura S, Shinomura Y, Matsuzawa Y (2001) Epidermal growth factor receptor mediates stress-induced expression of its ligands in rat gastric epithelial cells. Gastroenterology 120:108–116
8. Huang RP, Peng A, Golard A, Hossain MZ, Huang R, Liu YG, Boynton AL (2001) Hydrogen peroxide promotes transformation of rat liver non-neoplastic epithelial cells through activation of epidermal growth factor receptor. Mol Carcinog 30:209–217
9. Borrell-Pages M, Rojo F, Albanell J, Baselga J, Arribas J (2003) TACE is required for activation of the EGFR by TGF-α in tumors. EMBO J 22:1114–1124
10. Hirata A, Ogawa S, Kometani T, Kuwano T, Naito S, Kuwano M, Ono M (2002) ZD1839 (Iressa) induces antiangiogenic ef-

fects through inhibition of epidermal growth factor receptor tyrosine kinase. Cancer Res 62:2554–2560
11. Ciadiello F, Tortora G (2001) A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clin Cancer Res 7:2958–2970
12. Mendelsohn J, Baselga J (2000) The EGF receptor family as targets for cancer therapy. Oncogene 19:6550–6565
13. Ciardiello F, Caputo R, Bianco R, Damiano V, Fontanini G, Cuccato S, Placido S, De Bianco AR, Tortora G (2001) Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. Clin Cancer Res 7:1459–1465
14. Huang SM, Li J, Armstrong EA, Harari PM (2002) Modulation of radiation response and tumor-induced angiogenesis after epidermal growth factor receptor inhibition by ZD1839 (Iressa). Cancer Res 62:4300–4306
15. Raben D, Helfrich BA, Chan D, Johnson G, Bunn PA Jr (2002) ZD1839, a selective epidermal growth factor receptor tyrosine kinase inhibitor, alone and in combination with radiation and chemotherapy as a new therapeutic strategy in non-small cell lung cancer. Semin Oncol 29:37–46
16. Douglass EC (2003) Development of ZD1839 in colorectal cancer. Semin Oncol 30:17–22
17. Hiraoka S, Miyazaki Y, Kitamura S, Toyota M, Kiyohara T, Shinomura Y, Mukaida N, Matsuzawa Y (2001) Gastrin induces CXC chemokine expression in gastric epithelial cells through activation of NF-κB. Am J Physiol Gastrointest Liver Physiol 281:G735–G742
18. Digman JD, Lebovit RM, Roeder RG (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489
19. Noguchi H, Sakamoto C, Wada K, Akamatsu T, Uchida T, Tatsuguchi A, Matsui H, Fukui H, Fujimori T, Kasuga M (1999) Expression of Heregulin α, erbB2, and erbB3 and their influences on proliferation of gastric epithelial cells. Gastroenterology 117:1119–1127
20. Normanno N, Bianco C, De Luca A, Maiello MR, Salomon DS (2003) Target-based agents against ErbB receptors and their ligands: a novel approach to cancer treatment. Endocr Relat Cancer 10:1–21
21. Simizu S, Takada M, Umezawa K, Imoto M (1998) Requirement of caspase-3(-like) protease-mediated hydrogen peroxide production for apoptosis induced by various anticancer drugs. J Biol Chem 273:26900–26907
22. Lemjabbar H, Li D, Gallup M, Sidhu S, Drori E, Basbaum C (2003) Tobacco smoke-induced lung cell proliferation mediated by tumor necrosis factor alpha-converting enzyme and amphiregulin. J Biol Chem 278:26202–26207
23. Takenobu H, Yamazaki A, Hirata M, Umata T, Mekada E (2003) The stress- and inflammatory cytokine-induced ectodomain shedding of heparin-binding epidermal growth factor-like growth factor is mediated by p38 MAPK, distinct from the 12-O-tetradecanoylphorbol-13-acetate- and lysophosphatidic acid-induced signaling cascades. J Biol Chem 278:17255–17262
24. Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, Ullrich A (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature 402:884–888
25. Barnard JA, Graves-Deal R, Pittelkow MR, Dubois RN, Cook P, Ramsey GW, Bishop PR, Damstrup L, Coffey RJ (1994) Auto- and cross-induction within the mammalian epidermal growth factor-related polypeptide family. J Biol Chem 269:22817–22822
26. Huang S, Mills L, Mian B, Tellez C, McCarty M, Yang X-D, Gudas JM, Bar-Eli M (2002) Fully humanized neutralizing antibodies to interleukin-8 (ABX-IL-8) inhibit angiogenesis, tumor growth, and metastasis of human melanoma. Am J Pathol 161:125–134
27. Mukaida N, Mahe Y, Matsushima K (1990) Cooperative interaction of nuclear factor-κB- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. J Biol Chem 265:21128–21133
28. Naef M, Yokoyama M, Friess H, Buchler MW, Korc M (1996) Co-expression of heparin-binding EGF-like growth factor and related peptides in human gastric carcinoma. Int J Cancer 66:315–321
29. Ravid T, Sweeney C, Gee P, Carraway KL III, Goldkorn T (2002) Epidermal growth factor receptor activation under oxidative stress fails to promote c-Cbl mediated down-regulation. J Biol Chem 277:31214–31219
30. Konishi H, Tanaka M, Takemura Y, Matsuzaki H, Ono Y, Kikkawa U, Nishizuka Y (1997) Activation of protein kinase C by tyrosine phosphorylation in response to H_2O_2. Proc Natl Acad Sci U S A 94:11233–11237
31. Sun X, Wu F, Datta R, Kharbanda S, Kufe D (2000) Interaction between protein kinase C delta and the c-Abl tyrosine kinase in the cellular response to oxidative stress. J Biol Chem 275:7470–7473
32. Majumder PK, Mishra NC, Sun X, Bharti A, Kharbanda S, Saxena S, Kufe D (2001) Targeting of protein kinase C delta to mitochondria in the oxidative stress response. Cell Growth Differ 12:465–470
33. Cesaro P, Raiteri E, Demoz M, Castino R, Baccino FM, Bonelli G, Isidoro C (2001) Expression of protein kinase C beta1 confers resistance to TNF alpha- and paclitaxel-induced apoptosis in HT-29 colon carcinoma cells. Int J Cancer 93:179–184
34. Han Y, Han ZY, Zhou XM, Shi R, Zheng Y, Shi YQ, Miao JY, Pan BR, Fan DM (2002) Expression and function of classical protein kinase C isoenzymes in gastric cancer cell line and its drug-resistant sublines. World J Gastroenterol 8:441–445
35. Kitadai Y, Haruma K, Mukaida N, Ohmoto Y, Matsutani N, Yasui W, Yamamoto S, Sumii K, Kajiyama G, Fidler IJ, Tahara E (2000) Regulation of disease-progression genes in human gastric carcinoma cells by Interleukin 8. Clin Cancer Res 6:2735–2740
36. Bottero V, Busuttil V, Loubat A, Magnè N, Fischel J-L, Milano G, Peyron J-F (2001) Activation of nuclear factor κB through the IKK complex by the topoisomerase poisons SN38 and doxorubicin: a brake to apoptosis in HeLa human carcinoma cells. Cancer Res 61:7785–7791