Tumor-specific Cytotoxicity and Type of Cell Death Induced by Gefitinib in Oral Squamous Cell Carcinoma Cell Lines

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Abstract. *Gefitinib is an orally active, selective epidermal growth factor receptor-tyrosine kinase inhibitor. The present study was aimed at evaluating the antitumor activity of gefitinib alone or in combination with other antitumor agents. Gefitinib showed higher cytotoxicity against five human tumor cell lines (HSC-2, HSC-3, HSC-4, T98G and U87MG) than against three human normal oral cells (gingival fibroblast HGF, pulp cell HPC and periodontal ligament fibroblast HPLF). Gefitinib showed little or no growth stimulation effects at lower concentrations (so-called hormetic effect). Non-cytotoxic concentration of gefitinib effectively enhanced the cytotoxicity of docetaxel against HSC-2 and T98G cell, but failed to enhance the cytotoxicity of other antitumor agents (mitoxantrone, doxorubicin, methotrexate, cisplatin, sodium ascorbate, sodium fluoride) or herbal extracts (Drynaria baronii, Angelica sinensis and Cornus officinalis Sieb. et Zucc). Gefitinib alone and combined with docetaxel induced internucleosomal DNA fragmentation and caspase-3 activation in human promyelocytic leukemia HL-60 cells, but not in HSC-2 or T98G cells. Combination treatment with gefitinib and docetaxel induced the formation of acidic organelles (stained with acridine orange) and mitochondrial shrinkage, vacuolization and production of autophagosome and the loss of cell surface microvilli, without destruction of cell surface and nuclear membranes in HSC-2 and T98G cells (demonstrated by transmission electron microscopy), suggesting the induction of autophagy in HSC-2 and T98G cells.*

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Epidermal growth factor receptor (EGFR) is an important receptor involved in signaling pathways implicated in the proliferation and survival of cancer cells. EGFR is often highly expressed in human tumors, including oral squamous cell carcinomas (OSCC), and higher expression of EGFR is frequently accompanied by the development of malignant tumor (1-3).

EGFR and the cell cycle progression have been independently evaluated as targets for therapy, and there is evidence supporting a role for the inhibition of cell cycling through blockade of EGFR-mediated signals *via* small-molecule tyrosine kinase inhibitors (TKIs) for the cytosolic kinase domain, or by antibody targeting of the extracellular portions of EGFR (4-7).

Gefitinib (Iressa™, ZD1839) is a low molecular weight tyrosine kinase inhibitor (TKI) that competes for ATP binding to the catalytic kinase domain of EGFR, thus inhibiting EGF-stimulated receptor autophosphorylation and downstream signaling pathways. This ultimately results in the down-regulation of many cellular processes including proliferation, survival, migration and adhesion (8, 9).

Gefitinib inhibited the proliferation of OSCC cell lines in a dose- and time-dependent manner and led to cell cycle arrest with accumulation of cells in the G_1 phase, and a decrease of cells in S phase as determined by flow cytometric analysis (10). The up-regulation of CDKI in gefitinib-treated cell lines may be mediated by a p53-independent and heterogeneous ribonucleoprotein (hnRNP) C1/C2-dependent pathway (11). Gefitinib demonstrated the antiproliferative activity in various human cancer cell types *in vitro* (12) and potency against head and neck cancer (13), and treatment with gefitinib inhibited regional lymph metastasis in OSCC in an orthotopic nude mouse model (14, 15).

Although gefitinib can be used as an anticancer agent alone, more studies have focused on the combinative effects of gefitinib and other antitumor agents. The combination of other anticancer treatment given along with small-molecule TKIs has also been investigated. A cooperative *in vitro* antiproliferative effect was produced when cancer cells were treated with radiation followed by gefitinib. Combination treatment with radiation and gefitinib arrested OSCC cells at G_1 and G_2-M phases of the cell cycle with a decrease in the S phase population, and completely inhibited the downstream signaling pathway of EGFR (*i.e.* the DNA-dependent protein kinase complex pathways) (16). Addition of ZD1839 to cisplatin (CDDP) enabled CDDP treatment at a lower dose without compromising antiproliferative effects (17). Combination of mitoxantrone plus gefitinib caused a higher rate of apoptotic death of prostate cancer cells including enriched fraction of CD44 high cells (18). Combination of docetaxel with a cyclooxygenase-2 inhibitor and an EGFR TKI may further improve the efficacy of docetaxel and other taxane-based therapies in squamous cell carcinoma of the head and neck (19). *In vivo* tumor xenograft studies demonstrated that the combination of gefitinib and radiation caused growth inhibition and tumor regression of well-established OSCC tumors in athymic mice. Immunohistochemical analysis of OSCC xenografts revealed that gefitinib caused a striking decrease in tumor cell proliferation when combined with radiotherapy (16). Overall, these investigations have suggested that the inhibition of proliferation and invasion/migration in OSCC cell lines by gefitinib results in an anticancer effect *via* multiple cellular and molecular mechanisms. However, few previous studies have investigated the type of cell death induced by gefitinib in human OSCC cell lines.

The aim of this study was to evaluate the cytotoxicity, tumor specificity and combinatory effects of gefitinib with other antitumor agents and investigate the type of cell death induced by gefitinib in human OSCC cell lines. We have recently reported some tumor-specific cytotoxicity of three herbal extracts, *Drynaria baronii* (DB), *Angelica sinensis* (AS) and *Cornus officinalis* Sieb. et Zucc (CO) (20). Therefore, we also investigated here the combinatory effect of gefitinib and these herbal extracts.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), doxorubicin (Sigma Chem. Co., St. Louis, MO, USA); dimethylsulfoxide (DMSO), sodium fluoride (NaF), sodium iodide (NaI) (Wako Pure Chem. Ind., Ltd., Osaka, Japan); gefitinib (LC Laboratories®, PKC Pharmaceuticals, Inc., MA, USA); mitoxantrone (Kyowa, Tokyo, Japan); CDDP (Randa® Inj, Tokyo, Japan), docetaxel, sodium ascorbate (vitamin C; Tokyo Kasei Kogyo Co., Ltd., Tokyo); methotrexate (amethopterin; Nacalai Tesque, Inc., Kyoto, Japan). Hot-water extracts of herbs (DB, AS, CO) were prepared as described previously (20).

Table I. *Cytotoxic activity of gefitinib against cultured human normal and tumor cells. Tumor cells were inoculated at 2×10^3 cells in each well of 96-well microplates. Confluent normal cells were trypsinized and inoculated at a density of 1:4 of confluency. After incubation for 48 hours, the media were replaced with fresh media containing different concentrations of gefitinib, and the viable cell number was determined by MTT method after 48 hours. Each value represents the mean from 3 independent experiments (indicated as 1st, 2nd or 3rd). TS: Tumor specificity.*

	CC_{50} (μM)			
	1st	2nd	3rd	Mean±SD
Normal cells				
HGF	40.1	38.9	42.4	40.4±1.75
HPLF	42.7	39.2	40.7	40.8±1.74
HPC	36.9	29.1	38.1	34.7±4.88
(mean)				38.7
Tumor cells				
HSC-2	29.8	15.3	21.8	22.1±7.28
HSC-3	19.6	20.0	26.5	22.0±3.87
HSC-4	28.2	10.8	17.4	18.8±8.77
T98G	30.8	21.2	28.4	26.8±4.98
U87MG	35.9	21.4	32.3	29.9±7.55
(mean)				23.9
TS	1.62			

Cell culture. Human oral normal cells (HGF, HPC, HPLF) were prepared from periodontal tissues according to the guideline of the Intramural Board of Ethic Committee (No. A0808), after obtaining informed consent from the patients. Since these normal cells have a limited lifespan of about 20-30 population doubling level (PDL) due to *in vitro* senescence (21), cells at the 8-12 PDL were used for the present study. These normal cells, human OSCC cell lines (HSC-2, HSC-3, HSC-4) (kindly supplied by Professor Nagumo, Showa University), human glioblastoma cell line (T98G) and human glioma cells U87MG (kindly supplied by Dr. Iida, Showa University) were cultured in DMEM supplemented with 10% heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Human promyelocytic leukemic cell lines HL-60 (supplied by Professor Nakaya, Showa University) were cultured in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, as described previously (22).

Assay for cytotoxic activity. Near-confluent cells were treated for 48 hours with different concentrations of gefitinib in 96-microwell plates (Falcon®, Becton Dickinson and Company, Franklin Lakes, NJ, USA), and the viable adherent cell number was then determined by the MTT method, as described previously (22). The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve. The tumor-specificity index (TS) was measured by

the following equation: TS=(CC_{50}[HGF] + CC_{50}[HPC] + CC_{50}[HPLF]) / (CC_{50}[HSC-2] + CC_{50}[HSC-3] + CC_{50}[HSC-4] + CC_{50}[T98G] + CC_{50}[U87MG]) × (5/3) (Table I).

Assay for cytoxic activity combined with the other antitumor agents. Near-confluent HSC-2 and T98G cells were treated for 48 hours without (control), or with 10 μM gefitinib combined with different concentrations of the antitumor agents [mitoxantrone, doxorubicin (DXR), docetaxel, methotrexate (MTX), CDDP, sodium ascorbate (VC) and NaF] or herbal extracts (DB, AS, CO), and the viable adherent cell number and CC_{50} were determined as described above. We selected 48-hour incubation to detect both cytostatic and cytotoxic effect of test compounds. We found that 10 μM gefitinib partially reduced the viable cell number, and this concentration of gefinitib was used to detect the synergistic cytotoxicity with test compounds.

Assay for DNA fragmentation. HSC-2 or T98G cells (1.2×10^5) were inoculated onto a 6-well plate (9.6 cm^2) and incubated for 48 hours to allow complete adherence to the plate. Adherent HSC-2 or T98G cells and HL-60 cells freshly prepared (5×10^5) were cultured for 6, 24 or 48 hours (HL-60 cells: 6 hours) in fresh culture medium (3 ml) without (control) or with 1/2 CC_{50} of docetaxel combined with different concentrations (0, 5, or 10 μM) of gefitinib. Cells were then harvested, washed once with PBS (–) and lysed with 50 μl lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate solution]. The lysate was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 1-2 hours at 50˚C, and then mixed with 50 μl NaI solution (7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0), and then 200 μl of ethanol. After centrifugation for 20 minutes at 20,000 ×g, the precipitate was washed with 1 ml of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Sample (10-20 μl) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA molecular marker (Bayou Biolabs, Harahan, LA, USA) and DNA from apoptotic HL-60 cells induced by UV irradiation (6 J/m^2/min, 1 min) were run in parallel as positive controls (23). After staining with ethidium bromide, DNA was visualized by UV irradiation, and photographed by CCD camera (Bio Doc-It, UVP, Inc., Upland, CA, USA) (22).

Assay for caspase activation. HSC-2 or T98G cells (6×10^5) were inoculated on 85-mm dishes (Falcon Becton Dickinson) and incubated for 48 hours to allow complete adherence. The adherent HSC-2 or T98G cells and HL-60 cells freshly prepared (3×10^6 in 6-well plates) were further incubated for 6, 24 or 48 hours in fresh medium without (control) or with 1/2 CC_{50} of docetaxel combined with different concentrations (0, 5, or 10 μM) of gefitinib. Cells were washed with PBS(–) and lysed in the lysis solution [50 mM Tris-HCl (pH 7.5), 0.3% NP-40, 1 mM dithiothreitol]. After standing for 10 minutes on ice and centrifugation for 5 minutes at 21,000 ×g, the supernatant was collected. Lysate (50 μl, equivalent to 200 μg protein) was mixed with 50 μl lysis solution containing substrates for caspase-3 (DEVD-*p*-nitroanilide (*p*NA)), caspase-8 (IETD-*p*NA), or caspase-9 (LEHD-*p*NA). After incubation for 4 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by microplate reader (22).

Detection of acidic vesicular organelles with acridine orange staining. Acidic vesicular organelles were imaged by staining of the cells with 0.1 μg/ml acridine orange for 20 minutes. Samples were then examined under a Laser Scanning Microscope LSM510 (Carl Zeiss Inc., Gottingen, Germany) using the following filters: excitation filter 488 nm, emission filter 505-530 nm and >650 nm (22).

Electron microscopy. The cells were washed once with PBS, fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4˚C, and collected by scraping them off with a rubber policeman. Cells were then postfixed for one hour with 1% osmium tetraoxide-0.1 M cacodylate buffer (pH 7.4) at 4˚C, dehydrated and then embedded in Araldite 502 (CIBA-GEIGY, Basel, Switzerland). Fine sections were stained with uranyl acetate and lead citrate, and then observed under a JEM-1210 transmission electron microscope (JEOL) at an accelerating voltage of 100 kV (22).

Table II. *Hormetic effect of gefitinib against both human normal and tumor cells. The cells were inoculated and treated with gefitinib as described in Table I. Each value of three independent experiments (indicated as 1st, 2nd and 3rd) is shown.*

	Maximum response (% increase in the cell number at the maximum)			
	1st	2nd	3rd	Mean±SD
HGF	25.0	21.9	60.7	35.9±21.6
HPC	11.8	11.7	21.6	15.0±5.68
HPLF	70.3	25.3	32.6	42.7±24.1
HSC-2	10.3	0	21.7	10.7±10.9
HSC-3	0.94	0	17.9	6.27±10.1
HSC-4	0	0	0	0.00±0.00
T98G	10.3	0	0	3.43±5.95
U87MG	18.5	0	0	6.16±10.7

Results

Cytotoxic activity. Gefitinib displayed cytotoxicity against both human oral normal cells (HGF, HPC, HPLF) (mean CC_{50}=40.4, 34.7 and 40.8 μM, respectively) and human tumor cell lines (OSCC cell lines, HSC-2, HSC-3 and HSC-4: mean CC_{50}=22.1, 22.0 and 18.8 μM, respectively; human glioblastoma T98G: mean CC_{50}=26.8 μM; human glioma cells U87MG: mean CC_{50}=29.9 μM) after 48 hours' incubation. The cytotoxic effect of gefitinib was dose dependent. Gefitinib showed higher cytotoxicity against 5 human tumor cell lines, as compared with normal oral cells, yielding a tumor-specificity index (TS) of 1.62 (Table I).

Gefitinib showed little or no hormetic growth stimulation (0-10.1% of the maximum response) at wide ranges of lower concentrations in 5 human tumor cell lines. However gefitinib showed higher hormetic response (15.0-42.7% of the maximum response) in 3 human oral normal cell lines (Table II).

Combination effect of gefitinib and antitumor agents. Non-cytotoxic concentrations of geifitinib (both 5 and 10 μM) effectively enhanced the cytotoxicity of docetaxel

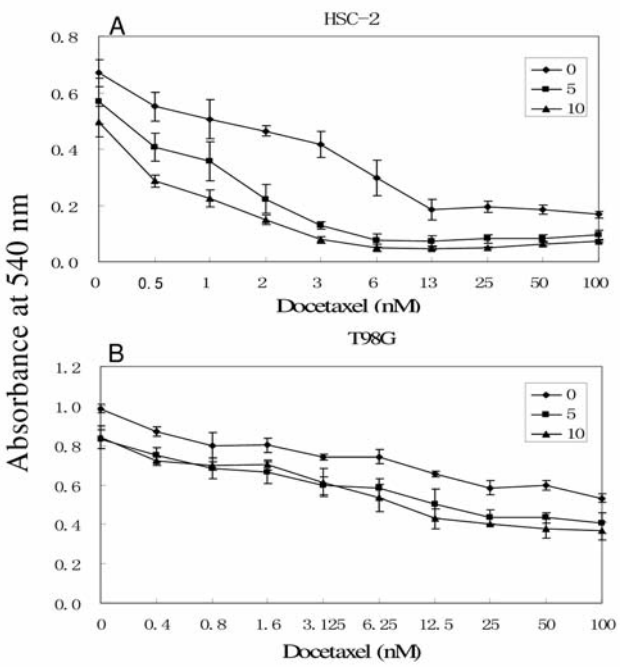

Figure 1. *Combinatory effect of gefitinib with docetaxel. Near confluent HSC-2 or T98G cells were incubated for 48 hours without or with 5 or 10 μM gefitinib, in combination with the indicated concentrations of docetaxel, and the viable cell number was then determined by MTT method. Each value represents the mean from triplicate assays.*

Table III. *Combination effect of gefitinib and docetaxel on the growth of HSC-2 and T98G cells. Near confluent HSC-2 and T98G cells were incubated for 48 hours without or with 5 or 10 μM gefitinib, together with the indicated concentrations of docetaxel. The relative viable cell number was then determined by the MTT method. Each value represents mean±SD from three independent experiments.*

	CC_{50} (nM) Docetaxel					
	HSC-2			T98G		
Gefitinib (μM)	0	5	10	0	5	10
1	5.14	1.23	0.76	>100	23.6	13.1
2	4.58	0.94	0.75	>100	21.9	13.5
3	5.26	0.71	0.66	>100	15.7	16.8
Mean	4.99	0.96	0.70	>100	20.4	14.5
±SD	0.36	0.26	0.06		4.16	2.02

against both HSC-2 and T98G cells. The mean CC_{50} of docetaxel without or combined with 5 or 10 μM gefitinib were: 4.99, 0.96 and 0.72 nM respectively in HSC-2 cells (Figure 1 A); and >100, 20.4 or 14.6 nM respectively in T98G cells (Figure 1 B) (Table III). However gefitinib failed to enhance the cytotoxicity of other antitumor agents (mitoxantrone, DXR, MTX, CDDP, sodium VC, NaF] or herbal extracts (DB, AS and CO) significantly (Table IV).

Table IV. *Combination effect of gefitinib and antitumor agents on the growth of HSC-2 and T98G cells. Near confluent HSC-2 and T98G cells were incubated for 48 hours without or with 10 μM gefitinib, together with the indicated concentrations of docetaxel, mitoxantrone (MITO), doxorubicin (DXR), methotrexate (MTX), cisplatin (CDDP), sodium ascorbate (VC), sodium fluoride (NaF), Drynaria baronii (DB), Angelica sinensis (AS) or Cornus officinalis Sieb. et Zucc (CO) extract. The relative viable cell number was then determined by the MTT method. Each value represents the mean±SD from three independent experiments.*

		CC_{50}	
		Gefitinib (0 μM)	Gefitinib (10 μM)
Docetaxel	HSC-2	4.99 nM	0.72 nM
	T98G	>100 nM	14.5 nM
MITO	HSC-2	69.7 nM	79.1 nM
	T98G	>125 nM	>125 nM
DXR	HSC-2	47.3 nM	65.3 nM
	T98G	>2000 nM	1788 nM
MTX	HSC-2	>250 μM	>250 μM
	T98G	>250 μM	>250 μM
CDDP	HSC-2	6.42 μM	5.86 μM
	T98G	49.9 μM	22.0 μM
VC	HSC-2	0.29 mM	0.29 mM
	T98G	2.47 mM	1.63 mM
NaF	HSC-2	2.21 mM	2.95 mM
	T98G	1.42 mM	1.39 mM
DB	HSC-2	>1600 μg/ml	>1600 μg/ml
	T98G	>1600 μg/ml	>1600 μg/ml
AS	HSC-2	>1600 μg/ml	>1600 μg/ml
	T98G	>1600 μg/ml	>1600 μg/ml
CO	HSC-2	>1600 μg/ml	>1600 μg/ml
	T98G	>1600 μg/ml	>1600 μg/ml

Induction of apoptosis. Untreated control HL-60 cells spontaneously produced a slight, but discernible amount of internucleosomal DNA fragmentation, possibly explaining their higher sensitivity to apoptosis inducers. Gefitinib (0, 5 or 10 μM) both with and without 1/2 CC_{50} docetaxel failed to induce internucleosomal DNA fragmentation in both HSC-2 and T98G cells, in contrast to the UV-induced apoptotic HL-60 cells, where the typical laddering pattern of DNA fragmentation was observed. (Figure 2). Gefitinib, both alone and in combination with 1/2 CC_{50} docetaxel, failed to activate caspase-3, -8 and -9 in both HSC-2 and T98G cells. It is notable that the extent of caspase-3 activation in both HSC-2 and T98G cells which had been treated with gefitinib, with or without docetaxel, was much lower than that of HL-60 cells which became apoptotic by UV irradiation (Figure 3).

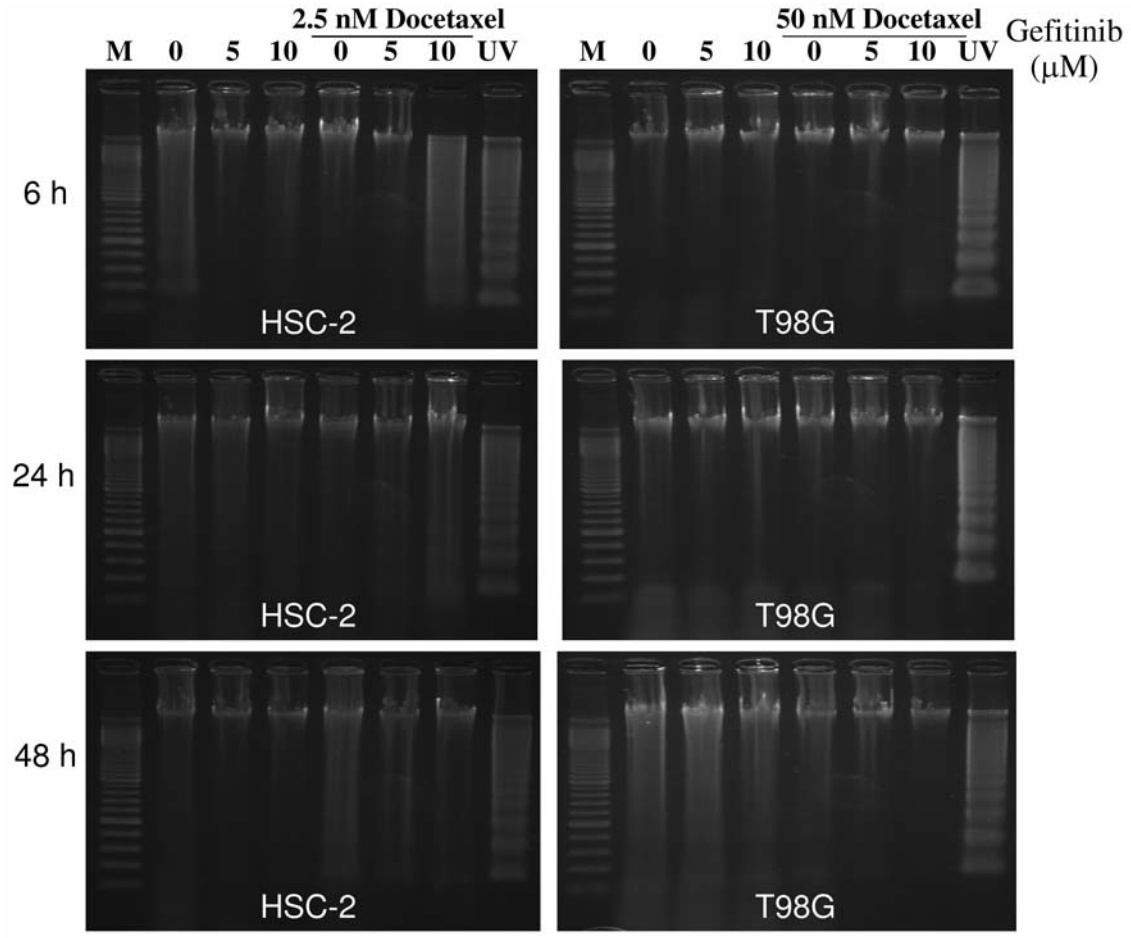

Figure 2. *Induction of DNA fragmentation by gefitinib combined with or without docetaxel. Near confluent HL-60, HSC-2 or T98G cells were incubated for 6, 24 or 48 hours with the indicated concentrations of gefitinib with or without 1/2 CC_{50} docetaxel (HSC-2: 2.5 nM; T98G: 50 nM). DNA was then extracted and applied to agarose gel electrophoresis. M: Marker DNA; UV, DNA from the HL-60 cells exposed to 1-min UV irradiation (22).*

Induction of autophagy. We next investigated whether gefitinib alone or combined with 1/2 CC_{50} docetaxel induced autophagy in HSC-2 and T98G cell lines. When these cells were induced to autophagy by incubating for 1 hour in HBSS, acidic organelles (stained by acridine orange) became apparent (Figure 4C, F). Treatment of HSC-2 cells for 6 hours with 5 μM gefitinib combined with 1/2 CC_{50} docetaxel (2.5 nM) enhanced the production of acidic organelles (Figure 4B), as compared with control (Figure 4A). Untreated T98G cells produced some acidic organelles (Figure 4D). Treatment of T98G cells for 6 hours with 5 μM gefitinib combined with 1/2 CC_{50} docetaxel (50 nM) also enhanced the production of acidic organelles (Figure 4E). These data suggest that gefitinib combined with docetaxel induced autophagy in HSC-2 and T98G cells.

Changes in cell ultrastructure. Electron microscopic analysis was performed to reveal the fine structural changes of HSC-2 and T98G cells cultured with gefitinib and docetaxel (Figure 5). When HSC-2 cells were cultured with gefitinib (5 μM) plus docetaxel (2.5 nM) for 6 hours, slight expansion of mitochondria accompanying the decrease in the number of cristae and electron density was observed (Figure 5B). When HSC-2 cells were cultured for 24 hours under the same conditions, equatorially arranged chromatin forming a double row and a lack of nuclear membrane were observed. Filamentous bundles surrounded the cytoplasmic chromatin (Figure 5C). These changes suggest that supplementation of gefitinib and docetaxel for short periods induces slight damage of mitochondria, whereas that for long periods induces the arrest of the mitotic process at metaphase.

In T98G glioblastomal cells cultured with gefitinib (5 μM) plus docetaxel (50 nM) for 6 and 24 hours, mitotic findings including cytoplasmic chromatin without nuclear membrane were also recognized (Figure 5E, 5F). In addition to the structural change of the nucleus, many secondary lysosomes

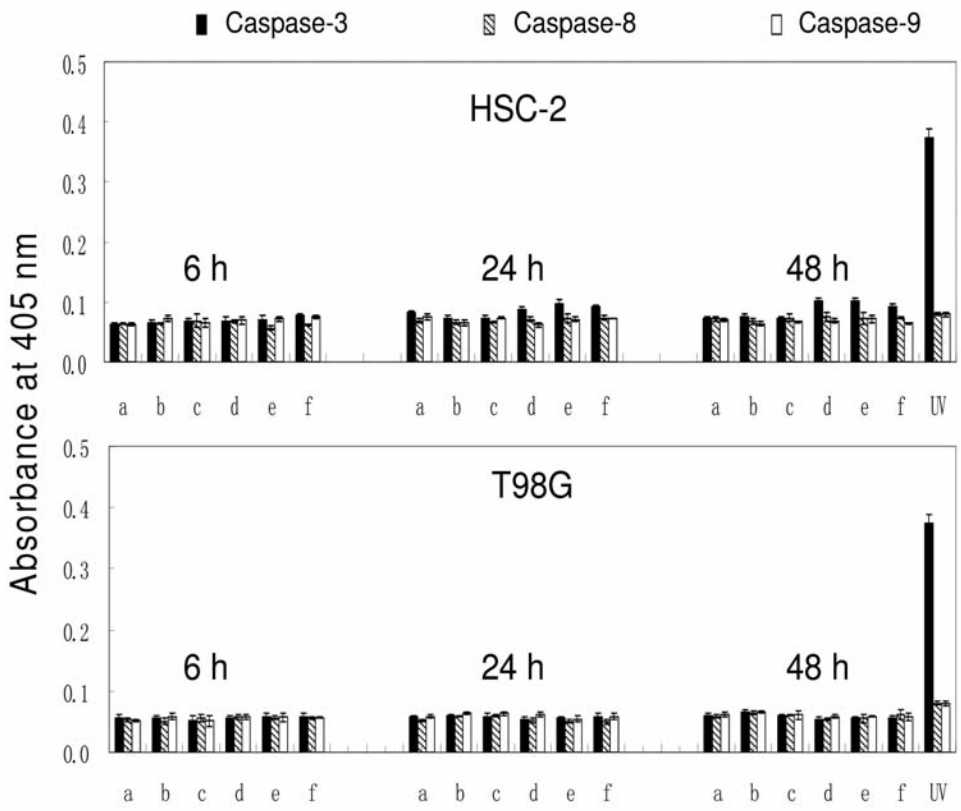

Figure 3. *Effect of gefitinib combined with or without docetaxel on caspase activity. Near confluent HSC-2 and T98G cells were incubated for 6, 24, or 48 hours with 0 (a, d), 5 (b, e) or 10 μM (c, f) gefitinib combined with (d, e, f) or without (a, b, c) 1/2 CC_{50} docetaxel (HSC-2: 2.5 nM; T98G: 50 nM). HL-60 cells were exposed to 1 min UV irradiation followed by 3 hours' incubation (22). Each point represents mean±S.D. from triplicate assays. UV: HL-60 cells were exposed to 1 min UV irradiation.*

containing autophagocytotic vesicles were prominent in the cytoplasm. These results indicate the supplementation of gefitinib to docetaxel induces cell division with an arrest of the mitotic process and autophagocytotic activity in glioblastomal cells.

Discussion

The EGFR is a key regulator of growth, differentiation, and survival of epithelial tumors. High expression of EGFR is frequently observed in many solid tumor types including OSCC (15). Gefitinib inhibition of EGF-stimulated receptor autophosphorylation and downstream signaling pathways ultimately results in the down-regulation of many cellular processes, including proliferation, survival, migration, and adhesion (11, 15, 16). The inhibition of proliferation and invasion/migration in OSCC cell lines by gefitinib results in an anticancer effect *via* multiple cellular and molecular mechanisms.

It has recently been reported that gefitinib can inhibit the proliferation of OSCC cell lines in a dose- and time-dependent manner and led to cell cycle arrest, with the accumulation of cells in the G_1 phase, and a decrease of cells in the S phase (24) or G_1 (11). Gefitinib also suppressed tumor metastasis in an animal model (14, 15). Gefitinib markedly reduced the ability of OSCC cells to adhere to fibronectin and the expression of integrins α3, α5, β1, β4, β5 and β6 (15). Cell growth was inhibited by an increase of the cell cycle inhibitor $p27^{kip1}$ and a decrease of its ubiquitin ligase subunit Skp2 (10).

The present study demonstrated that gefitinib displayed a higher antiproliferative effect on human OSCC cell lines than on human normal oral cell lines. The tumor specificity provided further evidence for the antitumor potential of gefitinib against OSCC cell lines. Many studies have demonstrated that the combination of gefitinib with other antitumor agents or treatments can further improve the efficacies of the latter (16-19). We investigated the cytotoxic activities of other antitumor agents and herbal extracts combined with gefitinib. We found non-cytotoxic concentrations of gefitinib (both 5 and 10 μM) effectively enhanced the cytotoxic action of docetaxel against both

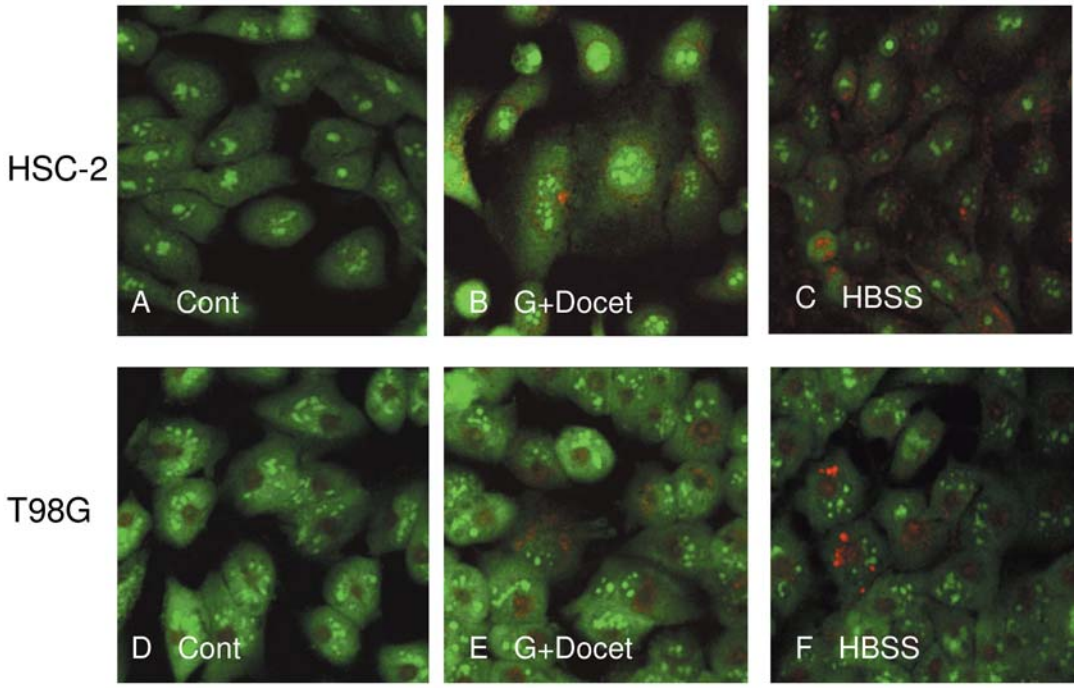

Figure 4. *Induction of the production of acidic organelles by gefitinib (G) combined with docetaxel (Docet). HSC-2 (A-C) and T98G (D-F) cells were incubated for 6 hours with DMEM+10% FBS (control)(A, D) or with 5 μM gefitinib combined with 1/2 CC_{50} docetaxel (HSC-2: 2.5 nM; T98G: 50 nM) in DMEM+10% FBS (B, E), or for 1 h in HBSS (C, F). The cells were then stained with acridine orange for the detection of acidic organelles.*

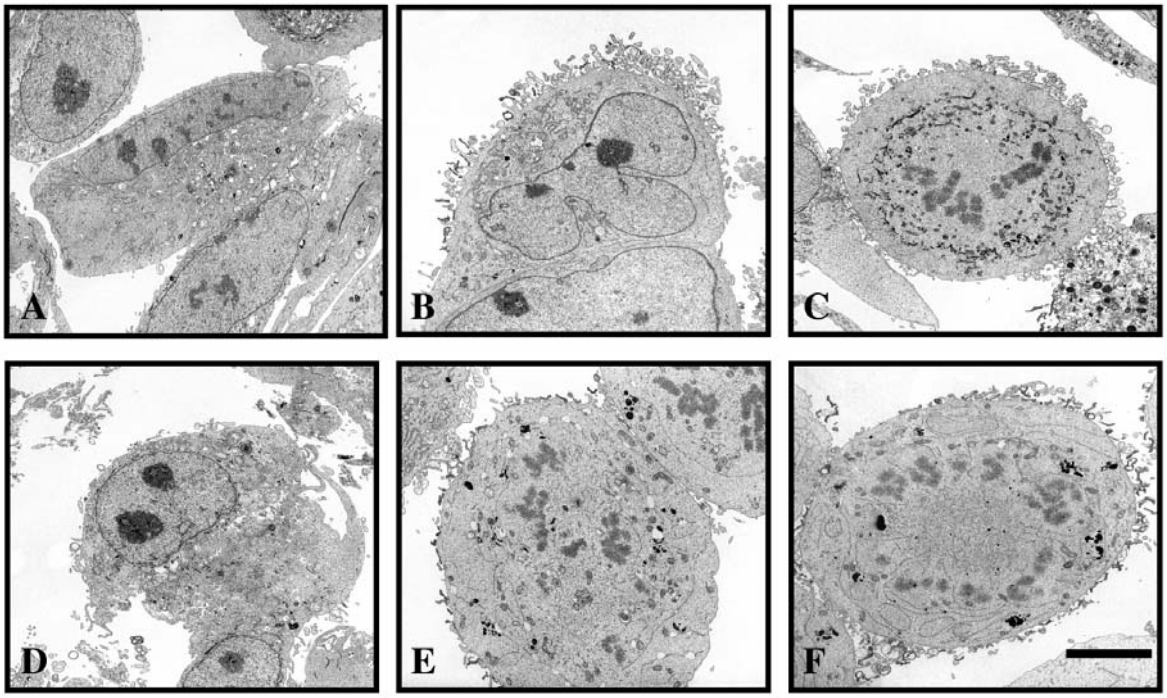

Figure 5. *Changes in the fine structure of HSC-2 and T98G cells after treatment with gefitinib combined with 1/2 CC_{50} docetaxel. HSC-2 (A, B, C) and T98G (D, E, F) cells were treated for 0 (control) (A, D), 6 (B, E) or 24 hours (C, F) with 5 μM gefitinib in the presence of 2.5 nM (for HSC-2) or 50 nM (for T98G) docetaxel, respectively, and were then processed for electron microscopy. Bar: 5 μm.*

HSC-2 and T98G cells, but failed to enhance the cytotoxic actions of the other antitumor agents (mitoxantrone, DXR, MTX, CDDP, VC, NaF) and herbal extracts (DB, AS, CO) significantly. This may be due to that the point of action of gefitinib is quite different from that of docetaxel that antagonizes the disassembly of β-tublin subunit of microtubules, in contrast to other antitumor agents that intercalate to DNA or inhibit DNA replication.

Next we investigated the antitumor mechanism of gefitinib combined with docetacel in HSC-2 and T98G cells. Gefitinib with or without docetaxel did not induce internucleosomal DNA fragmentation nor caspase activation, neither in HSC-2 nor in T98G cells, suggesting the induction of non-apoptotic cell death. This was consistent with findings of a previous paper (10). We found that treatment of HSC-2 and T98G cells for 6 hours with 5 μM gefitinib combined with 1/2 CC_{50} docetaxel induced the production of many secondary lysosomes containing the autophagocytotic vesicles, accompanied by an arrest of the mitotic process in both OSCC and glioblastomal cells.

The results here suggest gefitinib combined with docetaxel might induce autophagy but not apoptosis in HSC-2 and T98G cells. However, many studies have suggested that gefitinib was able to induce apoptosis in different tumor cells. Chang *et al*. found that gefitinib induced apoptosis in human lung cancer cells, and p53 played an important role (25). Pédebosq *et al*. found that gefitinib induced apoptosis in a concentration-dependent manner on human glioblastoma cells (26). Lindhagen *et al*. (27) reported that gefitinib had significant cytotoxic activity in acute myelogenous leukemia (AML) by inducing apoptosis through non-EGFR-dependent pathways. These data suggest that the type of cell death induced by gefitinib may be determined not only by the chemical structure of the inducer (28), but also by the drug-sensitivity or type of the target cell line, which is consistent with the conclusion of the study by Sebastian *et al*. (29).

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