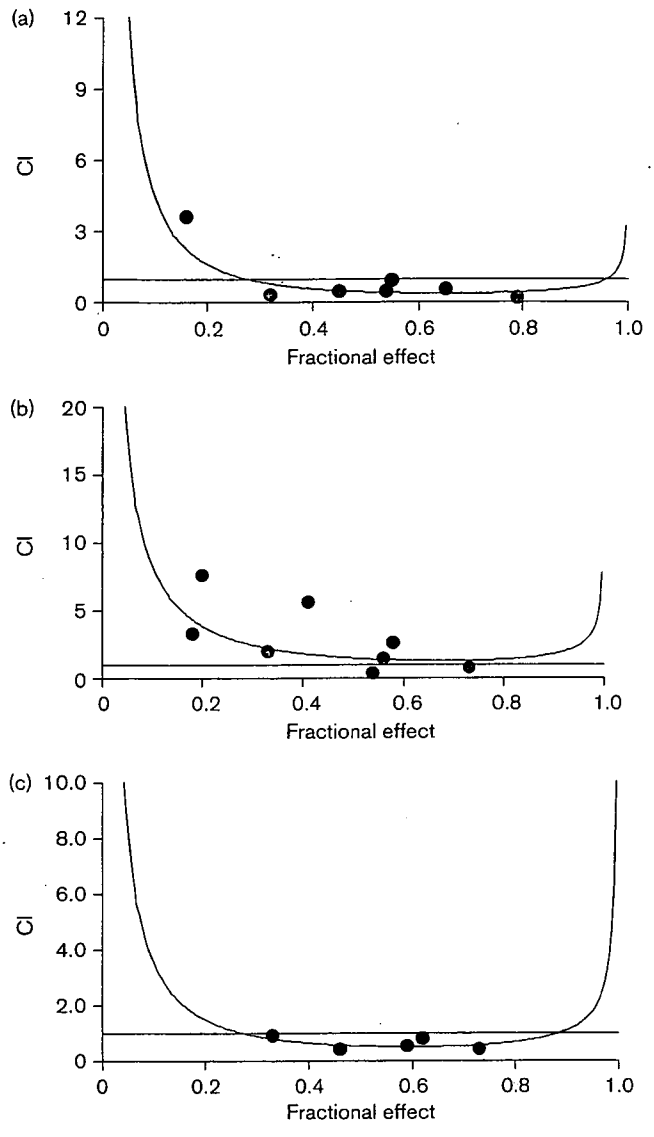
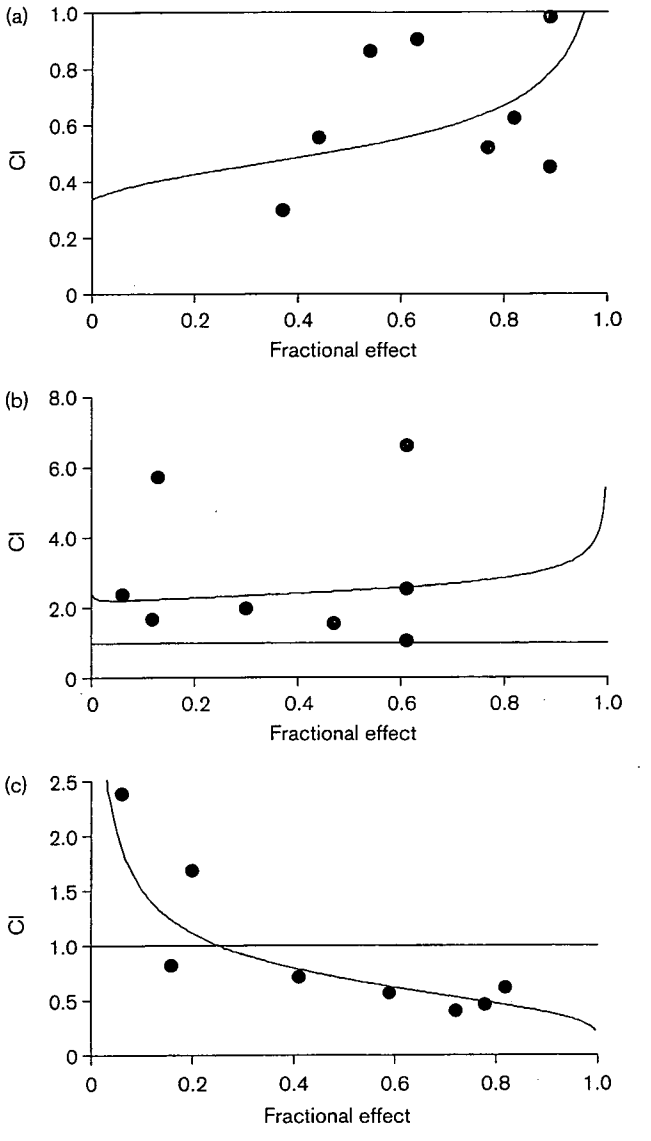
Fig. 2



CI of interactions between 5-FU and L-OHP in MKN-1 cells. Cells were treated with (a) 5-FU and L-OHP for 24 h simultaneously, (b) 5 FU for 24 h followed by L-OHP for 24 h or (c) L-OHP for 24 h followed by 5-FU for 24 h. The horizontal line indicates the level of 1.0 for the CI.

Fig. 3



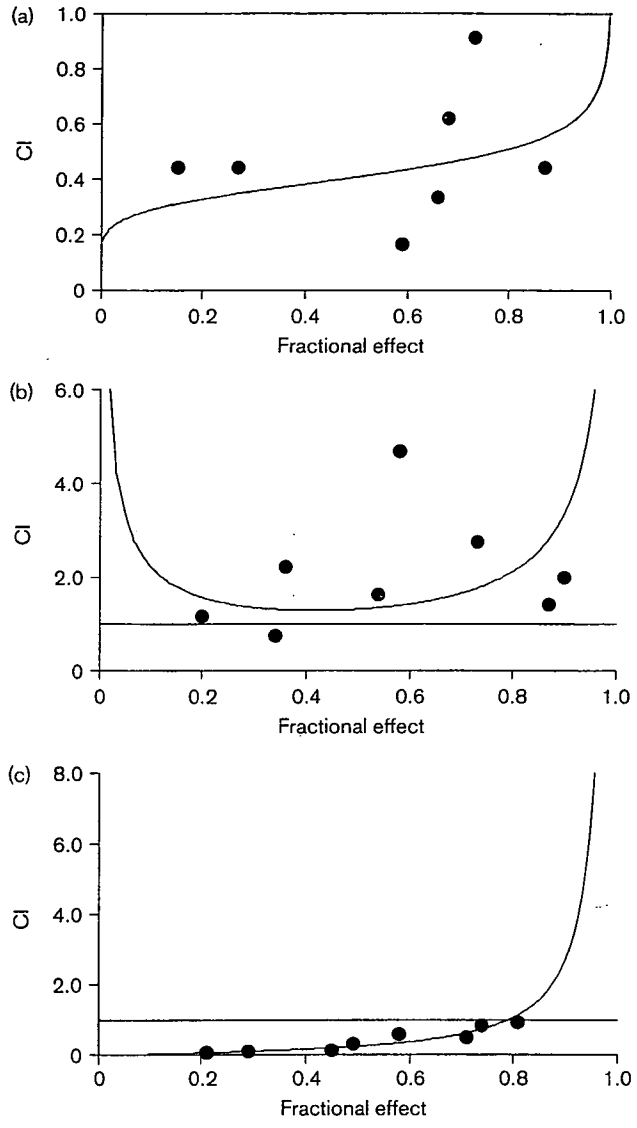
CI of interactions between 5-FU and L-OHP in NUGC-3 cells. Cells were treated with (a) 5-FU and L-OHP for 24 h simultaneously, (b) 5 FU for 24 h followed by L-OHP for 24 h or (c) L-OHP for 24 h followed by 5-FU for 24 h. The horizontal line indicates the level of 1.0 for the CI.

Conversely, when the reverse sequence was used, a distinct antagonism was observed (Fig. 5b).

Effect of 5-FU and L-OHP combination in long-term culture

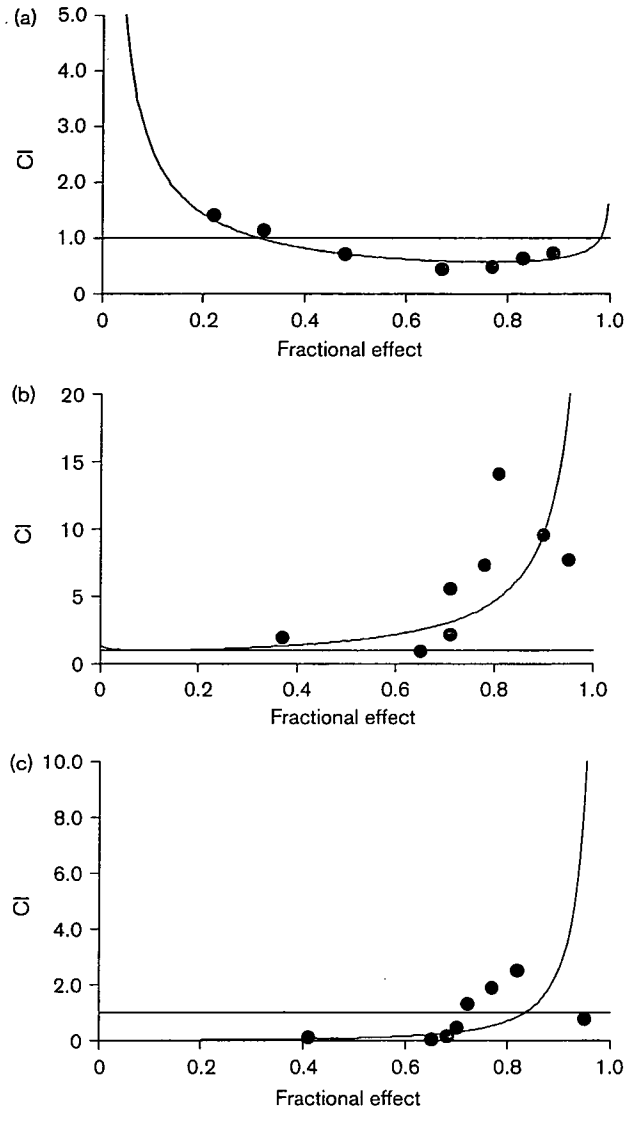
To confirm the results obtained by median-effect analysis, we compared the total number of cells 7 days after the same number of NUGC-3 cells (5×10^5) had been treated with various administration schedules of 5-FU and L-OHP at two fixed doses of 5-FU and L-OHP around the IC_{50} (Table 2). For both doses, the total cell numbers were lowest in simultaneous treatment, but there were no significant differences in the cell numbers between simultaneous treatment and the sequence L-OHP followed by 5-FU. Of note is that the number of cells in the sequence 5-FU followed by L-OHP was significantly higher than for other schedules. These data appeared to be consistent with those obtained by median-effect and apoptosis analyses.

Fig. 4



CI of interactions between 5-FU and L-OHP in NUGC-5 cells. Cells were treated with (a) 5-FU and L-OHP for 24 h simultaneously, (b) 5 FU for 24 h followed by L-OHP for 24 h or (c) L-OHP for 24 h followed by 5-FU for 24 h. The horizontal line indicates the level of 1.0 for the CI.

Fig. 5



CI of interactions between 5-FU and L-OHP in AZ-521 cells. Cells were treated with (a) 5-FU and L-OHP for 24 h simultaneously, (b) 5 FU for 24 h followed by L-OHP for 24 h or (c) L-OHP for 24 h followed by 5-FU for 24 h. The horizontal line indicates the level of 1.0 for the CI.

Cell cycle perturbation and apoptosis

In an attempt to explain the mechanisms underlying the different types of interaction, the effects of 5-FU and L-OHP on cell cycle distribution and apoptosis were investigated in AZ-521 cells (Table 3). The cells were treated with these drugs for 24 h either alone or in combination, with different schedules, and cell cycle distribution was analyzed 24, 36, 48, 72 and 96 h after treatment using flow cytometry. 5-FU alone at a dose of IC_{50} induced accumulation of cells in the G_0/G_1 phase, lasting until 96 h. At a dose of IC_{50}, L-OHP alone caused an increase in both G_0/G_1 and G_2/M populations, and G_0/G_1 arrest increased gradually until 96 h with the continuous decrease of G_2/M block. Treatment with 5-FU prior to L-OHP induced accumulation of cells in the G_0/G_1 phase, with an approximately similar distribution pattern to that observed in cells treated with 5-FU alone. In contrast, the schedule of L-OHP before 5-FU produced both G_0/G_1 and G_2/M block, with almost the same distribution pattern as that induced by L-OHP alone. These findings indicate that cell cycle distribution patterns with the sequential combinations could be mostly influenced by the initial drug administered. Interestingly, simultaneous exposure led to accumulation

Table 2 Effects of treatment schedules of 5-FU and L-OHP combination on total cell number after 7 days

Drug dose	Total cell number ($x10^4$)		
	5-FU+L-OHP	5-FU → L-OHP	L-OHP → 5-FU
5-FU 76.9 μmol/l	37.8 ±16.4 [1]	88.5 ±12.0 [2]	57.9 ±12.7 [2]
L-OHP 7.55 μmol/l	[3]	[3]	[3]
5-FU 231 μmol/l	25.0 ±3.39 [2]	42.2 ±8.44 [4]	30.6 ±5.37 [2]
L-OHP 25.2 μmol/l	[3]	[3]	[3]

5-FU, 5-fluorouracil; L-OHP, oxaliplatin.
*A fixed number of NUGC-3 cells (5×10^5) were seeded and exposed to 76.9 μmol/l 5-FU and 7.55 μmol/l L-OHP or 231 μmol/l 5-FU and 25.2 μmol/l L-OHP in three combination schedules. The total yield of cells was determined after 7 days of incubation from the initiation of treatment. Data are means ± SD of five independent determinations.
[1] $P<0.001$,
[2] $P<0.005$,
[3] $P>0.05$,
[4] $P<0.05$ by Student's *t*-test.

of cells in both G_0/G_1 and G_2/M phase – a pattern almost identical with that caused by L-OHP alone – indicating that L-OHP might have a dominant effect in cell cycle progression as compared to 5-FU or that 5-FU might take more time to exert its activity than L-OHP.

To define the cytotoxic activities of combination schedules, drug-induced apoptosis was studied after treatment of AZ-521 cells by measuring the sub-G_1 population. The presence of hypodiploid DNA (sub-G_1) is associated with cells undergoing apoptosis. As shown in Table 3, simultaneous treatment induced both G_0/G_1 and G_2/M blockade, with induction of 61.5% apoptosis in the treated cells. The induction rate of apoptosis by this combination was the highest among those induced by these combination schedules and much greater than that of 5-FU alone (1.61–26.9%) or L-OHP alone (1.9–20%). Sequential administration of L-OHP followed by 5-FU also caused both G_0/G_1 and G_2/M block with the apoptotic population of 55.5%. In contrast, the reverse sequence resulted in G_0/G_1 block and the apoptosis was 20% – not significantly different from that induced by 5-FU or L-OHP singly. These findings indicate that simultaneous treatment and sequential schedule of L-OHP followed by 5-FU exhibited synergistic interaction in inducing apoptosis, but that sequential administration of 5-FU followed by L-OHP is antagonistic. These results are consistent with those obtained by combination assays.

Discussion

In this study, we examined the sequence dependency of 5-FU and L-OHP combination in four human gastric cancer cell lines *in vitro*. Both simultaneous combination and sequential treatment of L-OHP followed by 5-FU exhibited synergistic effects in all four cell lines, with the most efficacious interaction observed in simultaneous combination, whereas the reverse sequence yielded a clear antagonism. This observation was confirmed by the experiment that compared the cell number 7 days after various treatment schedules. However, our results are not consistent with the report by Fischel *et al.* who showed that the clinically relevant L-OHP and 5-FU combination was synergistic whatever the tested schedules using human colorectal cancer cell lines [17]. The different exposure time of L-OHP may explain the difference. In our study, we incubated cells with L-OHP for 24 instead of 2 h, not only because the pharmacokinetics of L-OHP administered at dose of 130 mg/m^2 for 4 h showed that the plasma half-life of L-OHP was approximately 27 h [23], but also because more than 1.5 μg/ml (3.8 μmol/l) of total plasma concentration of platinum lasted at least 24 h

Table 3 Cell cycle perturbation and apoptosis (%) induced by 5-FU and L-OHP in AZ-521 cells

Treatment	24 h				36 h				48 h				72 h				96 h			
	G_0/G_1	S	G_2/M	Apo	G_0/G_1	S	G_2/M	Apo	G_0/G_1	S	G_2/M	Apo	G_0/G_1	S	G_2/M	Apo	G_0/G_1	S	G_2/M	Apo
Control	49.5	36.5	14.0	1.82																
5-FU	86.6	4.49	8.91	1.61	87.8	4.16	8.04	3.11	86.0	7.19	6.81	4.60	82.4	6.00	11.6	12.2	80.6	6.50	12.9	26.9
L-OHP	63.6	3.60	32.8	1.89	64.5	7.30	28.2	7.74	68.2	6.70	25.1	14.7	70.5	7.10	22.4	14.8	70.5	14.4	15.1	20.0
L-OHP + 5-FU	64.9	3.50	31.6	2.27	65.3	6.70	28.0	8.05	68.7	5.50	25.8	14.6	73.4	6.50	20.1	42.7	73.9	9.30	16.8	61.5
5-FU → L-OHP					87.3	5.29	7.41	3.93	86.5	5.96	7.54	7.73	85.7	7.68	6.62	20.0	85.6	7.74	6.66	16.2
L-OHP → 5-FU					64.7	8.80	26.5	7.23	68.3	5.80	25.9	13.7	72.5	6.70	20.8	37.7	73.4	10.0	16. 6	55.5

Cells were treated with 5-FU or L-OHP singly or in combination at the IC_{50} doses, and subjected to FACS analyses after collecting floating and trypsinized adherent cells at various times following drug exposure as described in Materials and methods. The apoptotic population percentages (Apo) were determined by measuring the sub-G_1 phase. The data presented are the mean percentage values from three independent experiments.

when patients were administered with 130 mg/m^2 of L-OHP for 2 h [24]. Therefore, the sequence and exposure time of administration of these drugs might be important in determining the extent of therapeutic synergy.

To elucidate the possible mechanisms underlying the synergistic interaction, we further analyzed the perturbations induced in the cell cycle by flow cytometric analyses using AZ-521 cells. First, we found that 24-h treatment with 5-FU markedly affected the cell cycle distribution, producing a clear accumulation in the G_0/G_1 phase and induced apoptosis in 26.9% of treated cells. L-OHP alone induced 20% of apoptosis by arresting cells in both G_0/G_1 and G_2/M phases. Simultaneous 24-h exposure to 5-FU and L-OHP and sequential 24-h exposure to 5-FU immediately after L-OHP treatment led to 61.5 and 55.5% apoptosis, respectively, without affecting cell cycle distribution induced by L-OHP. These results imply that 5-FU may kill the cells recovering from the mitotic block produced by L-OHP as they progress into S phase, accounting for the synergistic interaction. In contrast, 5-FU followed by L-OHP resulted in an antagonistic effect, reducing the rate of apoptosis to 20%. This would probably be explained by the decrease in the G_2 population targeted by L-OHP, because 5-FU pre-treatment caused accumulation of cells at the G_1/S boundary, thereby reducing the number of cells entering the G_2 phase.

In our study, simultaneous treatment and a sequential schedule of L-OHP followed by 5-FU exhibited synergistic interaction. It has been shown that cisplatin can inhibit methionine uptake into tumor cells, resulting in increased methionine synthesis and subsequent expansion of the reduced folate pool [25,26]. In the presence of 5-FU, these biochemical events lead to greater stabilization of the ternary complex formed between 5-fluoro-dUMP-thymidylate synthetase and 5-10-methylenetetrahydrofolate [27]. Such mechanisms may explain, at least in part, the synergistic interaction presently observed between L-OHP and 5-FU. In addition, recent pharmacokinetic investigations have suggested that L-OHP can alter 5-FU clearance [28]. L-OHP can inhibit dihydropyrimidine dehydrogenase, which is the rate-controlling enzyme of 5-FU catabolism [29]. Conversely, 5-FU may also influence L-OHP cytotoxic effects. Previously, we reported a significant reduction of the repair of cisplatin-induced DNA interstrand crosslinks in cells exposed to 5-FU/cisplatin [30], presumably through 5-FU-induced reduction of ERCC1 mRNA expression [31]. It is thus likely that 5-FU may induce similar molecular effects when combined with L-OHP. Moreover, experiments in colon cancer cell lines have demonstrated that L-OHP treatment results in downregulation of both thymidylate synthase (TS) mRNA level and free TS protein expression [32]. This TS modulation and downregulation may

provide a basis for explaining synergism in the simultaneous and sequence L-OHP followed by 5-FU treatment.

Unlike cisplatin, L-OHP appears to arrest cells at both the G_0/G_1 and G_2/M phases, indicating an action distinct from that of cisplatin, which causes an accumulation of cells in the G_2/M phase [33,34]. Therefore, the different patterns of DNA damage induced [35] and distinct cell cycle perturbations between L-OHP and cisplatin may induce different interactions with other drugs. Accordingly, 5-FU followed by L-OHP exhibited a clear antagonism, as opposed to the 5-FU and cisplatin combination, where sequential treatment of 5-FU followed by cisplatin shows a synergistic activity [36–39]. The sequence-dependent synergy exhibited by the 5-FU and cisplatin combination can be explained by the mechanism of DNA damage repair and detoxification processes, i.e. pre-treatment of 5-FU increased cisplatin cytotoxicity and even circumvents cisplatin resistance by inhibiting repair of platinum–DNA interstrand crosslinks as well as by reducing the cellular GSH levels [30,40]. For the combination with 5-FU followed by L-OHP, such mechanisms may not be involved. In spite of many similarities between L-OHP and cisplatin, there are important differences in their targets and mechanisms of action that may be related to their different activity profiles. Reciprocal interference of drug binding to nucleic acid might underlie this antagonism, since it has been shown that DNA binding of L-OHP is significantly reduced by the presence of 5-FU and *vice versa* [41].

Although the biochemical basis for the synergistic interaction between 5-FU and L-OHP remains to be elucidated, an antagonistic activity observed in the sequence 5-FU followed by L-OHP in a variety of human gastric cancer cell lines may provide a rationale for avoiding this sequence in clinical trials.

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ORIGINAL ARTICLE

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Activated Src and Ras induce gefitinib resistance by activation of signaling pathways downstream of epidermal growth factor receptor in human gallbladder adenocarcinoma cells

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Abstract *Purpose*: Although gefitinib, a selective inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase, has been demonstrated to exhibit its antitumor activity by the blockade of EGF receptor, the role of signaling pathways downstream of EGFR in gefitinib sensitivity remains unknown. In this study, we investigated the mechanistic role of Src and Ras, major oncogene products implicated in the pathogenesis of many human cancers in gefitinib sensitivity. *Methods*: Using parental and v-*src*- or c-H-*ras*-transfected HAG-1 human gallbladder adenocarcinoma cell lines, effects of gefitinib on cytotoxicity, cell cycle purtubation and apoptosis, and tyrosine phosphorylation of EGFR, Akt, and Erk were determined by WST-1 assay, flow cytometry, and Western blots, respectively. *Results*: Activated Ras and Src conferred a strong resistance to gefitinib by nearly 30-fold and 200-fold, respectively. Gefitinib induced accumulation of cells in the G0/G1 phase of the cell cycle at 24-h, with progressive expansion of apoptotic cell population in parental HAG-1 cells, but these effects were completely abolished in v-*src*- or c-H-*ras*-transfected cell line. Upon gefitinib treatment, EGFR activation and subsequent downstream activation through Erk and Akt were significantly inhibited in HAG-1 cells. By contrast, gefinitib failed to inhibit the activation of both Akt and Erk in v-src-transfected cells and Erk, but not Akt in c-H-ras-transfected cells, despite the blockade of EGFR activation in these respective cell lines. Treatment of v-src-transfected cells with herbimycin A, a Src tyrosine kinase inhibitor, partially reversed the gefitinib resistance, with concomitant inhibition of Akt and Erk. *Conclusion*: Our results suggest that activated Ras and Src could induce gefitinib resistance by activating either or both of Akt and Erk signaling pathways, thus providing a strategic rationale for assessment of these specific signaling molecules downstream of EGFR to customize treatment.

Keywords Gefitinib · EGFR · Akt · Erk · Apoptosis · Src · Ras

Abreviatons EGFR: Epidermal growth factor receptor · EGF: Epidermal growth factor · RTK: Receptor tyrosine kinase · MAPK: Mitogen activated protein kinase · Erk: Extracellular signal-regulated kinase · PI-3K: Phosphatidylinositol 3'-kinase

Introduction

Over the past decade, a variety of receptor tyrosine kinases have been identified to play a central role in the pathogenesis of various human cancers. Among these, epidermal growth factor receptor (EGFR) is overexpressed in a wide variety of epithelial malignancies including non-small cell lung, head, neck, colon, and breast cancers [1–4]. The overexpression of this receptor has been also detected in gallbladder cancer [5–7], a highly lethal disease with no known curative modality. Upon ligand binding, EGFR is activated through autophosphorylation by forming homodimerization or heterodimerization with other members of the HER family [8, 9], and transduces a variety of signals to downstream signal transduction cascades that lead to cellular proliferation and survival [10, 11]. Therefore, the inhibition of the EGFR signaling cascades may provide a rational therapeutic target of these chemotherapy-refractory cancers.

Gefitinib, a quinazoline derivative that inhibits EGFR tyrosine kinase activity, has been shown to be effective in

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preclinical studies and in late stages of clinical trials for non-small cell lung cancer [12, 13], although its activity appears not to be associated with the expression level of EGFR, but with the certain background of population, specific types of histology, and activating somatic mutations in the tyrosine kinase domain of EGFR [14–16]. This drug has been shown to inhibit major cell survival and growth signaling pathways such as Ras-Raf-Erk kinase pathway and phosphatidylinositol-3 kinase (PI-3K)-AKT pathway, as a consequence of inactivation of EGFR [17–20]. Conversely, persistent activity of the Ras/Erk and PI3K/Akt kinase pathways contributes to resistance of NSCLC cells to EGFR inhibitors [21–23]. Therefore, signaling molecules that activate these pathways might have the possibility to induce gefitinib resistance. The representative signaling molecules that share downstream signaling pathways with EGFR are Ras and Src, crucial cellular oncogene products implicated in the pathogenesis of many human cancers. Activation of Ras through gene amplification or point mutation was most frequently identified in a variety of human cancers, including adenocarcinoma of the pancreas, colon, and lung [24]. Ras transmits a signal to the serine/threonine kinase Raf, which subsequently activates mitogen-activated protein (MAP) kinase, resulting in cell proliferation through the transcriptional activation of a variety of targets, such as c-fos [25]. Activation of Src as detected by the elevation of tyrosine kinase activity was also identified in a variety of human cancers, such as breast, colon, skin, bladder, and pancreas [26]. Specifically, c-Src has been found to be highly activated in colon cancer metastasized to the liver [27]. Src phosphorylates a number of intracellular substrates on tyrosine residue [28], resulting in a generation of mitogenic and tumorigenic signals from Src to downstream signaling such as PI 3K-Akt and Ras-Raf-Erk kinase pathways.

Since the role of such oncogenic signalings in the gefitinib sensitivity remains to be clarified, we have investigated here the mechanistic role of Src and Ras, in gefitinib sensitivity, specifically through Akt and Erk pathways using parental and v-*src*- or H-*ras*-transfected HAG-1 human gallbladder carcinoma cell lines. In those cell lines showing EGFR-independent activity of the PI3K/Akt or Ras/Erk pathways, the relationship between the activity of these pathways and the ability of gefitinib to induce apoptosis was assessed.

Materials and methods

Cells and cultures and chemicals

HAG-1 is a human epithelial cell line derived from a moderately differentiated adenocarcinoma of the gallbladder [29]. No mutations and amplifications of H-, K-, or N-*ras* genes have been detected in this cell line. The HAG/ras5-1 cells were obtained by transfecting HAG-1 parental cells with activated c-H-*ras*, while HAG/src3-1 cells that express $p60^{v\text{-}src}$ protein were obtained by transfection of the pSV2/v-*src* into HAG-1 cells [30]. HAG/neo3-5 cells were obtained by transfection of HAG-1 cells with pSV2neo alone, which carries the gene for neomycin resistance. v-Src has a constitutively activated tyrosine kinase activity by the lack of negative regulatory domain. HAG-1 cells were cultured in Dulbecco's minimum essential medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), 100 UI/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. HAG/ras5-1 and HAG/src3-1 cells were grown in the same conditions, except that G418 (200 μg/ml) was added to the culture medium.

Gefitinib was kindly provided by AstraZeneca (Macclesfield, UK). Stock solutions were prepared in dimethyl sulfoxide (DMSO, Wako, Osaka, Japan) and stored at −20°C. The final concentration of DMSO for all experiments and treatments (including controls where no drug was added) was maintained at less than 0.02%. Herbimycin A was purchased from Wako Chimicals (Osaka, Japan). These conditions were found to be non-cytotoxic. Anti-EGF receptor, anti-MAPK, anti-Akt antibodies, and Protein A agarose were purchased from BD Biosciences (San Jose, CA USA).

Cytotoxicity, cell cycle analysis, and apoptosis measurement

The cytotoxic effect of gefitinib on HAG-1 cells was assessed by WST assay using manufacturer's instructions (DOJIN, Kumamoto, Japan). The WST assay is a colorimetric method in which the intensity of the dye is proportional to the number of the viable cells. Briefly, 100 μl cell suspension of HAG-1 cells was seeded into a 96-well plate at a density of 1,000 cells /well. After overnight incubation, 100 μl of gefitinib solutions at various concentrations were added. The effect of herbimycin A, a Src tyrosine kinase inhibitor, on the resistance to gefitinib was assessed in HAG/sec3-1 cells by co-treatment of gefitinib and herbimycin A. After incubation for 69 h at 37°C, 10 μl of solution A and solution B mixture was added to each well and the plates were incubated for a further 3 h at 37°C. Then the optical density was measured at 450 and 620 nm using an IMMUNO-MINI NJ-2300 spectrophotometer (Nalge Nunc International, Chester, NY, USA). Each experiment was performed using six replicate wells for each drug concentration and was carried out independently for three times. The IC_{50} value was defined as the concentration needed for a 50% reduction in the absorbance.

Control or gefitinib-treated cells were harvested by trypsinization, washed with PBS, and then fixed in 100% ethanol and stored at 4°C for up to 3 days prior to cell cycle analysis. After the removal of ethanol by centrifugation, cells were then washed with PBS and stained with a solution containing PI and RNase A on ice for 30 min. Cell cycle analysis was performed on a Becton Dickinson FACS/Calibur Flow Cytometer using the CELLQuest or

ModFit 3.0 software packages (Becton Dickinson, San Jose, CA USA), and the extent of apoptosis was determined by measuring the sub-G1 population.

Immunoprecipitation and Western blot analysis

The cells were washed twice with ice-cold PBS and scraped into 1 ml of radioimmunoprecipitation assay lysis buffer (50 mM Tris–HCl (pH 7.6), 300 mM NaCl, 0.4% (v/v) TritonX-100, 400 μM EDTA•2Na, 400 μM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$•$10H_2O$, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin). After removal of cell debris by centrifugation, protein concentrations of the supernatants were determined by using a BCA protein assay kit (Pierce, Rockford, IL). For Western blot, equal amounts of proteins or immunoprecipitated target proteins were resolved by 10% SDS-PAGE (polyacrylamide gel electrophoresis) and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA). Non-specific binding sites were blocked by incubating the membranes in blocking buffer (5% non-fat milk in 1 × TBS with 0.1% Tween-20) at room temperature for 1 h. The membranes were then incubated with primary antibodies against either phospho-EGFR (Tyr1068, Cell Signaling Technology), phospho-p44/42 MAPK (Thr202/Tyr204, Cell Signaling Technology), or phospho-Akt (Ser473, Cell Signaling Technology). The membranes were hybridized with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Immunoblots were developed with the enhanced chemiluminescence (ECL) system from Amersham Biosciences (Buckinghamshire, UK) and were then exposed to ECL hyperfilm according to the manufacture's instructions (Amersham Biosciences, Buckinghamshire, UK). The blots were striped and reprobed with primary antibodies against EGFR (2232, Cell Signaling Technology), MAPK (9102, Cell Signaling Technology), and Akt (9272, Cell Signaling Technology). For reblotting, membranes were incubated in stripping buffer (62.5 mM Tris/HCl, pH 6.8/2% (w/v) SDS/100 mM 2-mercaptoethanol) for 30 min at 50°C before washing, blocking, and incubating with antibody. Triplicate determinations were made in separate experiments.

Statistical analysis

The data were analyzed by the Mann-Whitney *U* test for statistical significance of the difference between groups. A *P* value of <0.01 was considered to indicate statistical significance.

Results

Effect of gefitinib on cytotoxicity of HAG-1, HAG/src3-1, and HAG/ras5-1 cells

To determine whether activated Src and Ras affect the gefinitib sensitivity, we examined the drug sensitivity in H-ras-transfected HAG/ras5-1 and v-src-transfected HAG/src3-1cells, and compared their IC_{50} values with those of parental HAG-1 cell line. The IC_{50} values of 72-h exposure of gefitinib were 0.12±0.05 μM for HAG-1 cells, 3.6±0.52 μM for HAG/ras5-1 cells, and 22±6.7 μM for HAG/src3-1 cells, indicating approximately 30-fold and 200-fold increases in resistance to gefitinib in HAG/ras5-1 and HAG/src3-1 cells, respectively, as compared with that of parental HAG-1 cells (Fig. 1).

Time course analysis of the effect of gefitinib on cell cycle progression and apoptosis

To examine whether the inhibitory effect observed in cytotoxicity assays reflect the arrest of cell cycle or apoptotic cell death, cells were treated with gefitinib for indicated times and the cell cycle progression and apoptosis was evaluated after PI staining by fluorescence-activated cell sorting analysis. When HAG-1 parental cells were treated with gefitinib at a dose of 1 μM, the proportion of cells in a G0/G1 phase increased from 60 to 87 % at 24 h from the beginning of the treatment, with corresponding decrease in cells in S and G2-M phase and reached a plateau afterward. The percentage of sub-G0/G1 cell population became evident after (72 h, 20%) 72 h post-treatment and progressively increased upon further treatment (96 h, 34%; 120 h, 50%) (Fig. 2a). Because cells in the sub-G0/G1 population represent apoptotic cells, the cytotoxicity by the treatment of gefitinib appeared to be due to progressive expansion of apoptotic cell population. By contrast, when HAG/ras5-1 or HAG/src3-1cells was treated with the same concentration of gefitinib, neither arrest of cells in the G0/G1 phase nor the sub-G0/G1 cell population became evident with incubation times in both cell lines (Fig. 2b, c).

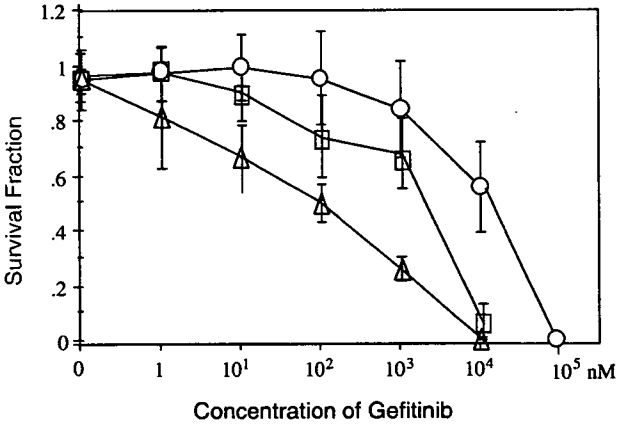

Fig. 1 Cytotoxicity of gefitinib against HAG-1 (*open triangle*), HAG/ras5-1 (*open square*), and HAG/src3-1 (*open circle*) cells. Cells were treated with various concentration of gefitinib for 72-h and assessed for cytotoxicity by WST-1 assay as described in Materials and methods. The data represent the means from three independent experiments. Bars, SD

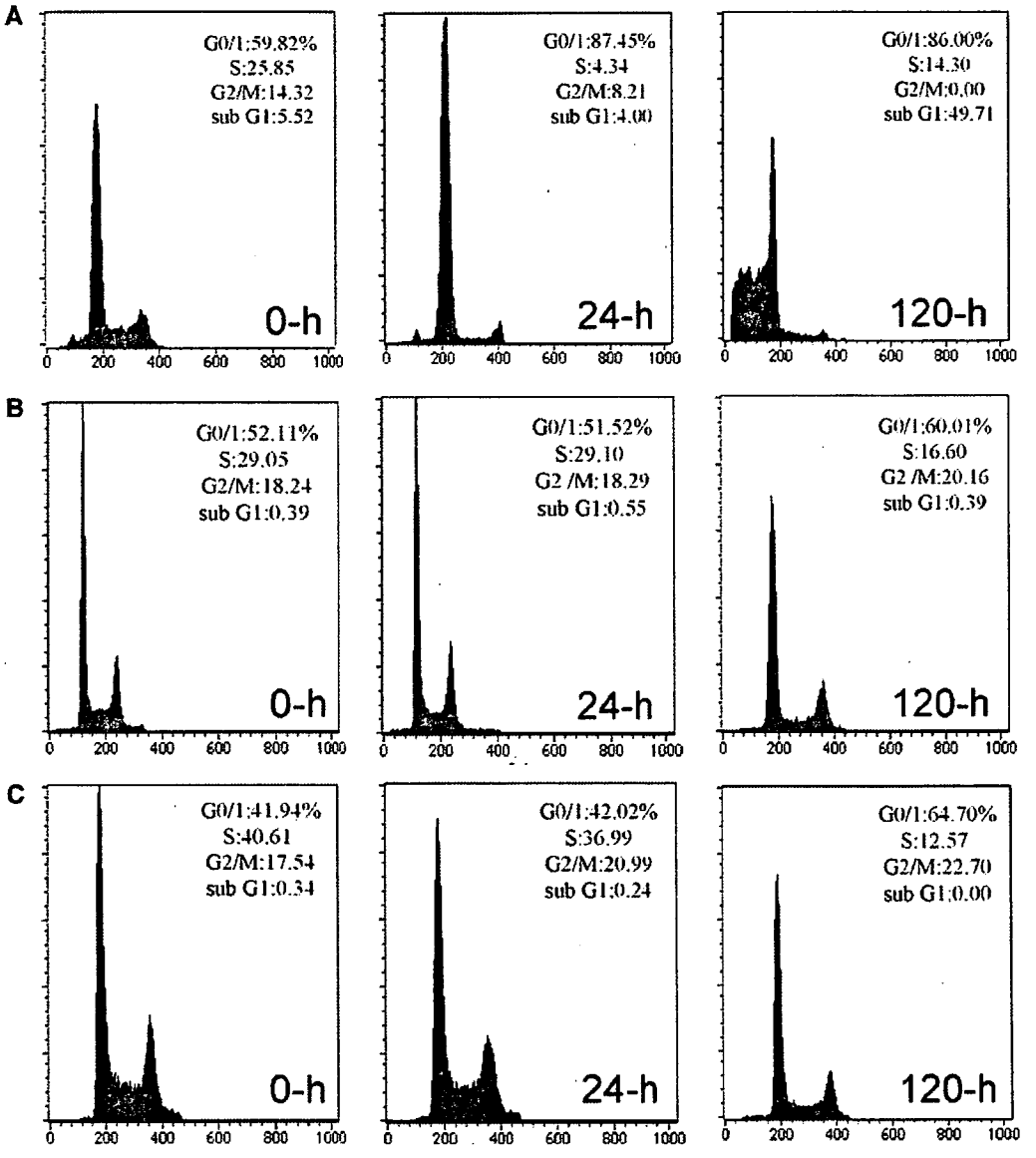

Fig. 2 Time course analysis of the effect of gefitinib on Cell cycle progression and apoptosis. HAG-1 (**a**), HAG/ras5-1 (**b**), and HAG/src3-1 (**c**) cells were stained with propidium iodide after exposure to gefitinib (1.0 μM) for 0, 24, and 120 h and analyzed by flow cytometry. Percentages of the total cell population in the different phases of cell cycle were determined with curve fitting using the ModFit 3.0 software. The mean values for each phase of the cell cycle are shown on the top right of each panel. Representative results of at least three experiments are shown

Inhibition of tyrosine phosphorylation of the EGFR by gefitinib in parental HAG-1, HAG/ras5-1, and HAG/src3-1 cells

To demonstrate the effect of gefitinib on the EGFR activation, we examined the expression and activation of EGFR in these three cell lines. As shown in Fig. 3, phosphorylated EGFR at tyrosine was detected without EGF stimulation in all three cell lines. When parental HAG-1 cells were treated with 1 μM gefitinib, constitutive phosphorylation of EGFR was significantly inhibited at 2-h post-treatment and remained to be suppressed over 24 h, without changing the relative amount of EGFR. Similarly, in HAG/ras5-1 cells, the same concentration of gefitinib completely suppressed the constitutive phosphorylation of EGFR at 2-h post-treatment and remained to be suppressed over 24 h. In HAG/src3-1 cells, however, inhibition of gefitinib on the constitutive phosphorylation of EGFR appears to be modest, declining gradually over 12-h post-treatment, with subsequent recovery to the initial level at 24 h.

Effects of gefitinib on autophosphorylation of Akt and Erk in parental HAG-1, HAG/ras5-1, and HAG/src3-1 cells

To demonstrate the effect of gefitinib on signaling pathways downstream of EGFR, we examined the expression

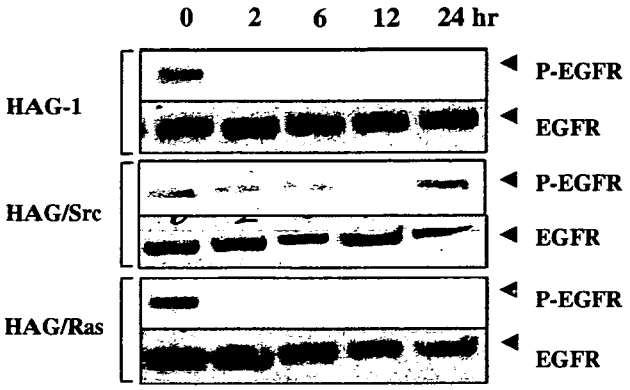

Fig. 3 Effect of gefitinib on the phosphorylation of EGFR in HAG-1, HAG/ras5-1, and HAG/src3-1 cells. Cells were exposed to 1 μM of gefitinib and incubated for indicated times. Western blots are shown for phospho- and total EGFR

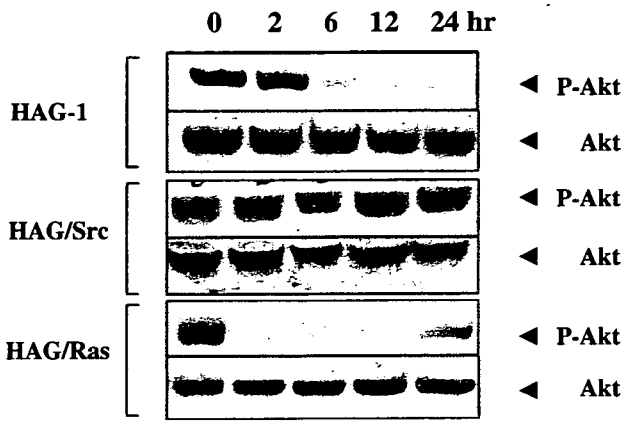

Fig. 5 Effect of gefitinib on the phosphorylation of AKT in HAG-1, HAG/ras5-1, and HAG/src3-1 cells. Cells were exposed to 1 μM of gefitinib and incubated for indicated times. Western blots are shown for phospho- and total Akt

and activation of Erk and Akt in these three cell lines. As shown in Fig. 4, tyrosine phosphorylation of Erk was seen in all the cell lines. Upon treatment with gefitinib, the phosphorylation of Erk was significantly suppressed only in parental HAG-1 cells, but was never suppressed in HAG/src3-1 and HAG/ras5-1 during the incubation periods. As shown in Fig. 5, tyrosine phosphorylation of Akt exhibited by HAG/src3-1 cells was never inhibited by gefitinib treatment during the incubation period. By contrast, these constitutive activations of Akt were significantly inhibited by gefitinib in parental HAG-1 cells. Of note, the activation of Akt is similarly inhibited in H-ras-transfected HAG/ras5-1 cells, indicating that activated Ras could not drive Akt activation in these cells.

Effects of Src kinase inhibitor on gefitinib resistance and Src-induced Akt/Erk tyrosine phosphorylation

To determine whether Src kinase activity is responsible for resistance to gefitinib in v-src-transfected cells, we studied the effect of herbimycin A on the gefitinib resistance in mock-transfected (HAG/neo3-5) and v-src-transfected (HAG/src3-1) cell lines. Combined treatment with gefitinib and 50 ng/ml of herbimycin A did not alter the sensitivity of gefitinib in HAG/neo3-5 cells, but significantly reduced gefitinib resistance in v-src-transfected HAG/src3-1 cells (Fig. 6). In v-src-transfected cells, gefitinib did not affect the phosphorylation status of both Akt and Erk (Fig. 7a), but exhibited its inhibitory activity against Akt and Erk phosphorylation when co-treated with herbimycin A (Fig. 7b). These data suggest that gefitinib resistance observed in HAG/src3-1 cells might be induced by Src kinase activity through activation of Akt and Erk.

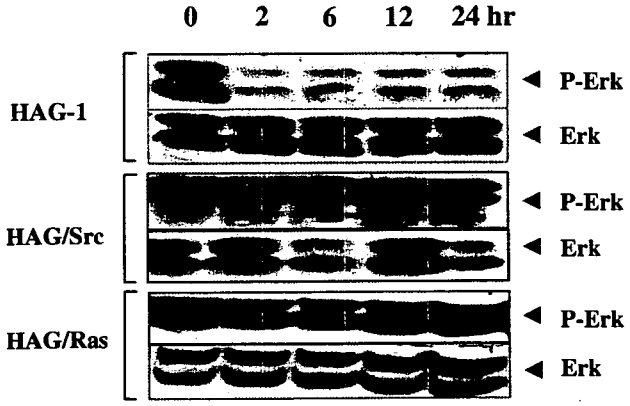

Fig. 4 Effect of gefitinibb on the phosphorylation of ERK in HAG-1, HAG/ras5-1, and HAG/src3-1 cells. Cells were exposed to 1 μM of gefitinib and incubated for indicated times. Western blots are shown for phospho- and total Erk

Discussion

In the present study, we found that the IC_{50} of gefitinib against HAG-1 cells was 0.12 μM for 72 h exposure, a comparable IC_{50} concentration exhibited by highly sensitive A431 squamous carcinoma cell line [21]. Using this gefitinib-sensitive cell line, we examined the role of activated Ras and Src in the gefitinib resistance after transfection with activated c-H-*ras* or v-*src*, since these oncogenes are major signaling molecules that share downstream signaling pathways with EGFR and closely associated with the pathogenesis of many human cancers. We found that activation of Ras and Src conferred a strong resistance to gefitinib by nearly 30-fold and 200-fold, respectively, and abolished completely its apoptosis-inducing activity. Moreover, v-Src-induced gefitinib resistance was partially reversed by the Src kinase inhibitor, indicating a potential role of Src tyrosine kinase activity in inducing gefitinib resistance. Recently, activating K-ras mutations has been shown to be specifically detected in gefitinib-resistant cells, suggesting that the occurrence of K-Ras mutations is correlated with

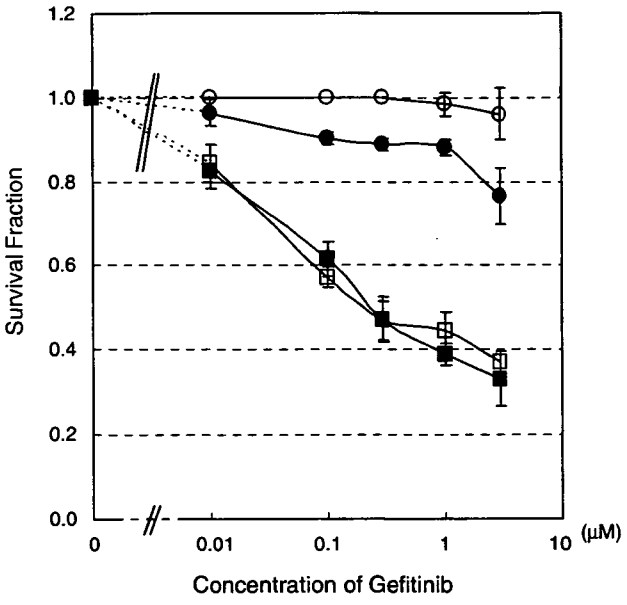

Fig. 6 Effects of herbimycin A on the cytotoxicity of gefitinib in HAG/neo3-5 and HAG/src3-1 cells. Cells were treated with various concentration of gefitinib with or without 50 ng/ml of herbimycin A and assessed for cytotoxicity by WST-1 assay as described in Materials and methods. HAG/neo3-5 cells with (*filled square*) or without (*open square*) herbimycin A. HAG/src3-1 cells with (*filled circle*) or without (*open circle*) herbimycin A. The data represent the means from three independent experiments. Bars, SD

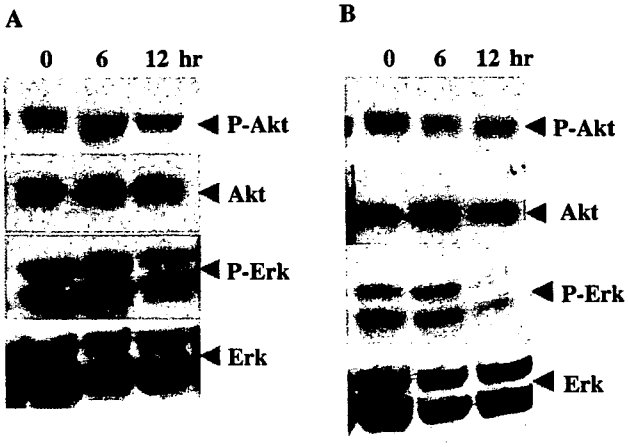

Fig. 7 Effects of gefitinib on the phosphorylation of Akt and Erk in HAG/src3-1 cells. The cells were exposed to 1 μM of gefitinib alone (**a**) or in combination with 100 ng/ml of herbimycin A (**b**) and incubated for indicated times. Western blots are shown for phospho- and total Akt and Erk

resistance to EGFR antagonists [31]. Taken together, these data suggest that concomitant presence of either of these activated oncogenes could induce a strong resistance to gefitinib.

In the downstream of EGFR, there are two major cell survival and growth signaling pathways, i.e., Ras/Raf-1/Erk pathway and PI-3K-AKT pathway. Recently, it has been reported that simultaneous inhibition of both Ras/Raf-1/Erk and PI-3K/AKT pathways are important for the execution of gefitinib-induced antiproliferative effect and apoptosis, and that persistent activity of either of these signaling pathways is involved in the decreased or lack of sensitivity to EGFR inhibitor [21, 32]. Therefore, we examined the activity of these signaling pathways by measuring the activity of EGFR, Akt, and Erk following treatment with gefitinib. Upon gefitinib treatment, tyrosine phosphorylation of EGFR was significantly inhibited in all three cell lines, although the inhibition was modest in v-src-transfected cell line. Lower inhibition of phosphorylated EGFR in v-src transfected cells might be explained by the direct phosphorylation of EGFR by Src kinase because the physical association between Src and EGFR [33] and direct phosphorylation of EGFR on tyrosine 845 [34] have been reported. Activation of ERK and AKT was significantly inhibited in HAG-1 parental cells, followed by accumulation of cells in the G0/G1 phase of the cell cycle, with progressive expansion of apoptotic cell population. However, neither arrest of cell cycle nor apoptosis was evident in both v-*src*- and c-H-*ras*-transfected cell lines. Moreover, gefitinib failed to inhibit the phosphorylation of both Akt and Erk in v-*src*-transfected cells and Erk, but not Akt in c-H-*ras*-transfected cells. These data suggest that activated Src can induce gefitinib resistance by activating both PI-3K-AKT and Ras-Raf-Erk pathways and that activated Ras induce gefitinib resistance by activating Ras-Raf-Erk pathway alone. Accordingly, herbimycin A partially reversed the resistance to gefitinib, with concomitant inhibition of Akt and Erk in v-src-transfected cells. These data suggest that gefitinib resistance might be induced by activating either or both of these Akt and Erk signalings. Similar observations have been reported in non-small cell lung cancer cell lines, indicating that simultaneous inhibition of PI-3K/Akt and MEK/Erk reduces tumor cell survival more effectively than inhibition of each pathway alone [21]. Recent immunohistochemical study in patients with chemotherapy-refractory non-small cell lung cancer showed that positive expression of phosphorylated Erk is significantly associated with poor response to gefitinib [35]. In our experiments, however, a strong resistance was observed with concomitant activation of Akt and Erk rather than a single activation of Erk, indicating that Akt activation would also be crucial for gefitinib resistance.

Of note, in this study, the activated c-H-Ras could not activate the Akt, suggesting that the signaling pathway from c-H-Ras to PI-3K-Akt might not operate in the HAG-1 cell line. This is in contrast to the previous studies showing that mutant K-Ras and H-Ras preferentially activate the Ras/Erk pathway and the PI3K/Akt pathway, respectively [36–38]. Therefore, it is suggested that preferential signaling downstream of Ras may vary from cell to cell, controlling elegantly the cell growth and survival depending on cell types. Likewise, the mechanism of gefitinib resistance cannot be explained by the

activation of PI-3K/Akt and/or Ras/Erk pathways. Src phosphorylates a number of intracellular substrates associated with cell growth and survival. The signaling pathway downstream of EGFR other than Ras/Raf-1/Erk and PI-3K/Akt is Janus tyrosine kinase (Jak)/signal transducers and activators of transcription (STAT) pathways. In our previous study, the Jak2/Stat3 has been shown to be activated in v-Src-transfected HAG-1 cells [39]. Therefore, activation of Stat3 might be involved in gefitinib resistance in these cells. We are currently investigating this possibility.

In recent study, strong correlations have been reported between EGFR mutations and improved response and survival in patients with non-small cell lung cancer, who have been treated with gefitinib [40]. Therefore, assessment of EGFR mutations is currently recommended to customize treatment. Likewise, the search for activated oncogenes such as Src and Ras as well as identification of signaling molecules downstream of EGFR would be beneficial for prediction of clinical response to gefitinib, and combination with specific inhibitors against Src, Ras, MEK, or PI-3K might become useful to overcome gefitinib-resistance.

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Lecture

解　説

大腸がんに対する抗体療法の最近の進歩*

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Key Words：EGFR, cetuximab, VEGF, bevacizumab, panitumumab

はじめに

がんを抗体で叩くという治療は，実はそう新しいものではない．1975年にハイブリドーマ技術を用いた細胞融合により単クローン抗体産生が可能となり，抗体によるがん治療の試みが多数行われた．1980年代初頭には，悪性リンパ腫に対する抗イディオタイプ抗体[1]や，大腸がんに対するA7-NCS[2]などが実際に臨床で使用された．「魔法の弾丸」あるいは「ミサイル療法」の名を冠されたこれらの治療法は，やがて消えてゆくことになる．治療法として思ったほどの効果が得られなかったためであるが，腫瘍での標的抗原の不均一さや，免疫担当細胞の病巣内浸潤性の低さ，そしてヒト抗マウス抗体反応(human anti-mouse antibody reaction；HAMA)の誘導などが原因であった．当時投与されていた抗体は，標的とするタンパクを免疫したマウスから作られた抗体であり，体内へ投与されても容易に排除された．例外的に大腸がんに対する17-1A抗体はドイツで臨床開発が進められたが，抗体療法ががん治療の表舞台にカムバックするまでには1997年に抗CD20キメラ抗体であるrituximabがFDAに認可されるのを待たなくてはいけなかった．分子生物学的手法の進歩により，抗体の定常領域をヒト由来に置き換えたキメラ抗体や，相補性決定部位のみマウスであるヒト化抗体，すべての部分でヒト由来のアミノ酸配列を有したヒト抗体が登場し，抗体そのものの抗原性を低下させることで，HAMAを抑制し，より長期投与が可能となった．その後の抗体療法の隆盛は言わずもがなであるが，1998年にtrastuzumabが乳がんに，2000年にgemtuzumabは急性骨髄性白血病に，2002年と2003年にはiburitumomab, situmomabが悪性リンパ腫に，1998年にはinfliximabが慢性関節リウマチに対しての薬剤としてFDAに承認された．このように，がん以外にも抗体療法は活用されているが，大腸がんに対するcetuximabとbevacizumabがFDAに承認されたのは，2004年と比較的新しい．

抗体療法の進歩

抗体療法が以前より進歩した点としては，①抗体そのものの免疫原性の克服，②抗体に抗がん剤や放射線同位元素，毒素などを抱合させ，局所での殺細胞効果を高めた抗体の作製，③分子量を少なくし，腫瘍への浸透性を高めた単鎖抗体(single chain Fv；scFv)，複数のscFvを結合させた多価抗体(multivalent antibody)の作製，などがあげられる．

Morrisonらにより開発されたキメラ抗体は，既存のマウス抗体の重鎖と軽鎖の定常領域をヒト抗体の定常領域に置き換えたものである(図1)[3]．これは，主にマウス抗体の定常領域によりHAMAが誘導されているため，これを克服するための策であるが，可変領域は手を加えていないため，抗体の抗原に対する親和性は変化しないまま，抗体自体の免疫原性を低下させた点で優れてい

* Recent progress of antibody therapy for colorectal cancer.

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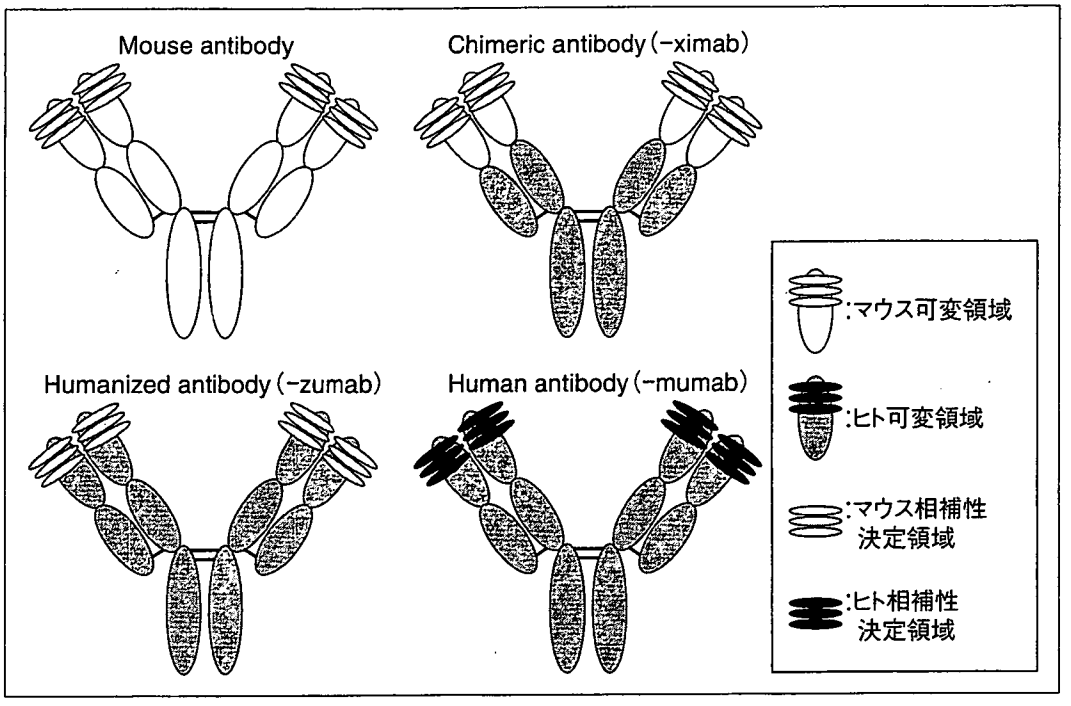

図1 抗体の進歩

た．Winterらはさらに可変領域にある相補性決定部位(complementarity determining region；CDR)であるCDR1, CDR2, CDR3の3か所のみマウス抗体由来の配列を用い，それ以外をすべてヒト抗体由来に置換したヒト化抗体を開発した(図1)[4]．これにより90%以上ヒト由来のアミノ酸配列をもつ抗体が作られ，免疫原性の克服という問題についてはほぼ目的を達成したが，さらに100%ヒト由来のアミノ酸配列をもつ抗体も登場した(図1)．これは，ヒト免疫グロブリン遺伝子を導入したトランスジェニックマウスや，ファージディスプレイを用いて作られる[5]．これらの技術革新により，以前は24時間程度であった血中半減期も2週間程度と長くなり，HAMAの誘導もほぼ抑えられるようになり，抗体療法という治療法が確立された．

抗体が，抗腫瘍効果を発揮する機序としては，①腫瘍細胞表面のレセプターへの結合によるリガンド結合の阻害，またはリガンドへの結合によるレセプターへの結合阻害，②腫瘍への抗体結合後，抗体のFc領域により誘導される補体による細胞障害(補体依存性細胞障害活性：complement-dependent cytotoxcity；CDC)，③同様にFc領域により誘導される免疫担当細胞による細胞障害(抗体依存性細胞障害活性：antibody-dependent cell-mediated cytotoxcity；ADCC)，④抗イディオタイプ反応を利用した腫瘍抗原に対する免疫担当細胞の感作，などいくつかのものが推察されている(図2)．EGF受容体に対する抗体であるcetuximab, matuzumab, panitumumabは主に②，③により，リガンドであるVEGFに対する抗体であるbevacizumabは①の作用機序により抗腫瘍効果を発揮する．

現在多数の疾患に対して抗体薬剤が臨床導入されているが，その半数以上は悪性腫瘍に対するものである(表1)．今後さらにその数は増加すると予測される．

上皮細胞増殖因子EGFR (epidermal cell growth factor receptor) を標的にした抗体療法

1．がんとEGFR

EGFRは分子量170KDaの受容体型チロシンキナーゼであり，さまざまな正常組織や形質転換組織で細胞増殖を促進する．EGFRはErbB(HER)ファミリーと呼ばれる受容体型チロシンキナーゼの一つである．ErbB(HER)ファミリーは1から4まであり，ErbB1(HER1)がEGFRである．EGFRはリガンド(EGF, TGFαなど)の刺激によりホモダイマーあるいはErbB2とのヘテロダイマー

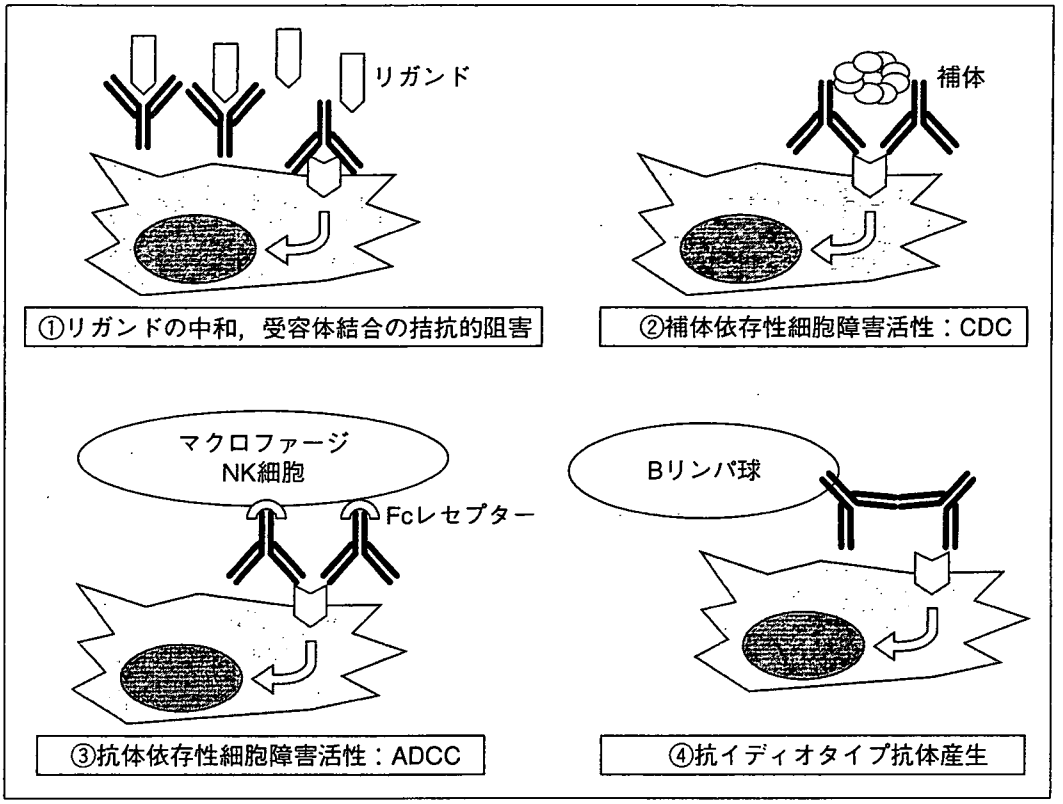

図2　抗体による抗腫瘍活性の機序

を形成し細胞内のチロシンキナーゼ(TK)が自己リン酸化を起こし，シグナルを下流へ伝える働きをもつ[6]．EGFRは多くの正常なヒト組織に発現しているが，多くのがんで過剰発現していることが知られており，大腸がん25～77%，食道がん43～89%，頭頸部がん80～100%，前立腺がん40～80%，膵がん30～89%，腎細胞がん50～90%，非小細胞肺がん40～80%，神経膠腫40～63%と報告されている．これらEGFRの過剰発現は，血管新生の誘導や，アポトーシス阻害と関連しており，シグナルの遮断により腫瘍の増殖を抑制できると考えられた．EGFR抗体はEGFRに結合し，抗体が結合したEGFRは細胞質内へ内在化(internalize)される．これにより細胞表面のEGFR数が減少し，結果としてリガンドの結合とその後の2量体の形成(dimerization)ならびにシグナル伝達を抑制する．Gefitinibやerlotinibなどのチロシンキナーゼ阻害剤はダイマーを形成したのちのEGFRの自己リン酸化を阻害することで，シグナルを遮断するといわれている(図3)．EGFR抗体は，担がんマウスモデルで腫瘍増殖抑制を示し[7]，EGFFRをターゲットにした分子標的治療薬開発が盛んに行われた．

現在いくつかのEGFRをターゲットにした抗体薬剤が開発中あるいはすでに使用中であり，それぞれについて解説を加える．

2．Cetuximab(IMC-C225)(Erbitax®)

CetuximabはEGFRをターゲットにしたヒト-マウスキメラIgG1抗体である．EGFRへの親和性は内在性のリガンドよりも1対数単位高いため，競合的に拮抗作用を示す．Cetuximabの投与により細胞周期阻害，アポトーシス誘導，血管新生阻害，転移浸潤抑制が認められ[8][9]，これが抗腫瘍効果につながっていると考えられている．担がんマウスでの実験では，イリノテカンとの併用による相乗効果や，イリノテカン不応となった後にcetuximabとイリノテカンを併用することで，抗腫瘍効果が再び認められることが示され[10]，臨床試験に期待が集まった．

まずEGFR陽性イリノテカン不応大腸がん患者57名に対してcetuximab単剤投与が行われた[11]．PRとなった症例は5名(8.8%)であったが，これは免疫染色でのEGFRの発現強度と相関は認められなかった．また，有害反応として，grade 3/4

表1 現在臨床導入されている抗体療法とその適応

がん種	抗 原	抗 体	タイプ	臨床試験
非ホジキンリンパ腫	CD20	Rituximab	キメラ	2001日本承認
	CD20	^{90}Y-ibritumomab tiuxetan	マウス＋放射性同位元素	2002FDA承認
	CD20	^{131}I-tositumomab	マウス＋放射性同位元素	2003FDA承認
	CD22	Epratuzumab	ヒト化	Phase Ⅰ-Ⅱ
	CD22	^{90}Y-epratuzumab	ヒト化＋放射性同位元素	Phase Ⅰ
	HLA-DR	Remitogen	ヒト化	Phase Ⅱ
急性骨髄性白血病	CD33	Gemtuzumab ozogamicin	ヒト化＋抗がん剤	2000FDA承認
B細胞性慢性リンパ球性白血病	CD52	Alemtuzumab	ヒト化	2001FDA承認
大腸がんなど	EGFR	Cetuximab	キメラ	2004FDA承認
	EGFR	Panitumumab	ヒト	Phase Ⅲ
	EGFR	h-R3	ヒト化	Phase Ⅰ/Ⅱ
大腸がん，肺がんなど	VEGF	Bevacizumab	ヒト化	2004FDA承認
大腸がん(術後)	Epithelial cellular-adhesion molecule	Edrecolomab	マウス	1995ドイツ承認
乳がんなど	HER2/neu	Trastuzumab	ヒト化	2001日本承認
上皮がんなど	CEA	^{90}Y-CEA-cide	ヒト化＋放射性同位元素	Phase Ⅰ/Ⅱ
固形腫瘍	VEGFR2	IMC-1C11	キメラ	Phase Ⅱ/Ⅲ
大腸がん	A33	huA33	ヒト化	Phase Ⅰ/Ⅱ
腎細胞がん	G250/MN	G250	キメラ	Phase Ⅰ/Ⅱ
上皮がん	Lewis Y抗原	SGN-15	キメラ	Phase Ⅰ/Ⅱ
		Hu3S193	ヒト化	Phase Ⅰ
メラノーマ	GD3	KW-2871	キメラ	Phase Ⅰ

(文献[37]より改変)

のアクネ様皮疹が16%，アレルギー反応が5%，倦怠感が4%に認められたが，皮疹のgradeによる解析では，grade 0, 1-2, 3-4の皮疹が起こった群それぞれの生存期間中央値は1.9か月，6.4か月，9.5か月であり，症例数は少ないながらも皮疹が抗腫瘍効果の指標となりうる可能性を示した．

BOND studyではEGFR陽性イリノテカン不応大腸がん患者をランダム化し，cetuximab単独群と，cetuximab＋イリノテカン群とで比較した[12]．抗腫瘍効果は奏効率が単独群で11%，併用群で23%と有意に併用群で優れていた(表2-a, b, c)．有害事象では併用群において，より下痢，倦怠感，アクネ様皮疹，好中球減少などが多く発現した．BOND studyでも同様に，皮疹が起こった症例は起こらない症例に比べて生存が長い傾向が示され，これは単独群，併用群ともに認められた．

BOND2 studyはBOND studyと同様の対象患者(イリノテカン不応)に対してイリノテカン＋cetuximab＋bevacizumab併用群(CBI)と，cetuximab＋bevacizumab併用群(CB)に分けて比較した試験である[13]．BOND studyのそれぞれのarmにbevacizumabを上乗せした格好だが，CBI群とCB群の奏効率はそれぞれ37%と20%であり，無増悪生存期間はそれぞれ7.9monと5.6monであった．この試験の意義は，cetuximabの有効性というよりも，分子標的治療薬同士の併用によりさらなる効果が期待できることを示したことにあった．しかし，そのことは同時に医療費の高騰という問題が身近になっていることを示唆していた．

Cetuximabは，初回治療例に対しても，既存の抗がん剤との併用により高い抗腫瘍効果を示している(表3)．これらの結果は，いずれもphase Ⅱのものであり，臨床の現場に取り入れられるには現在行われているphase Ⅲの結果を待たなくてはいけない．

臨床で用いる場合に注意すべき有害事象は，皮疹と点滴時の過敏症である．皮疹はEGFR阻害剤であるgefitinibと同様に，いわゆるアクネ様皮疹が特徴的であるが，75%に出現し，grade 3以上になる割合は，単剤投与で5.2%，イリノテカ

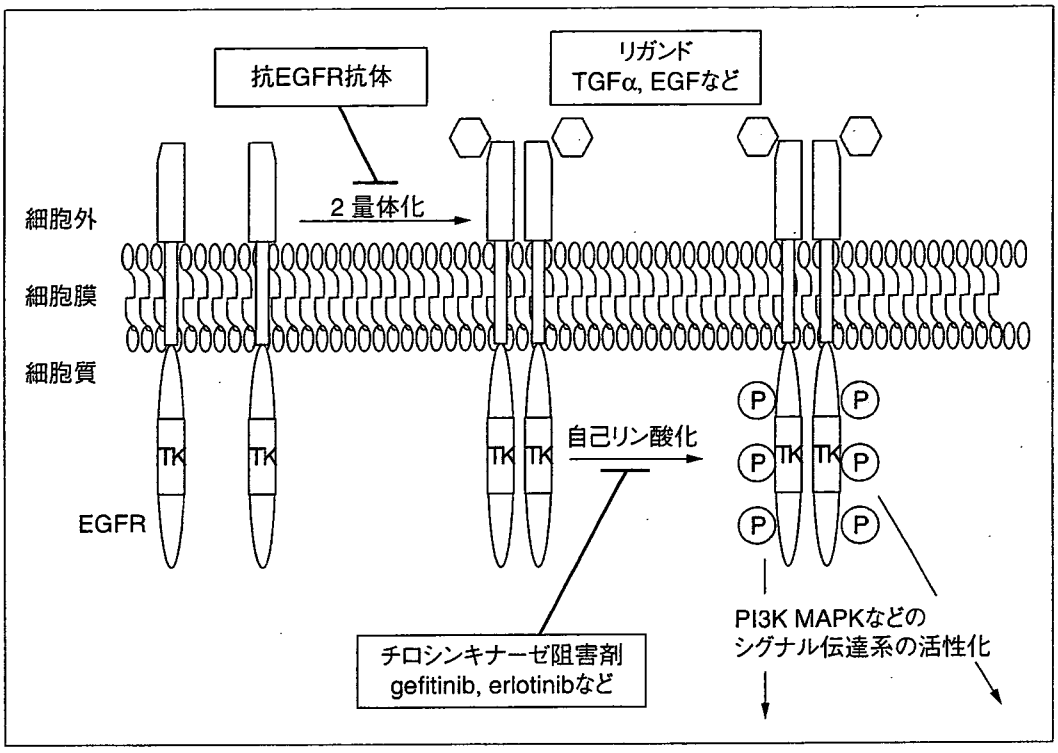

図3　EGFRと活性化のメカニズム(文献[36]より引用改変)

ンとの併用で9.3%と報告されている[14]. Grade 2までの場合，ビタミンDやミノマイシンの投与，メトロニダゾールクリームなどの塗布を行うが，grade 3以上の場合には休薬が勧められる．また，点滴時の過敏反応(infusion reaction)は，初回投与時に出ることが多く，その症状は微熱，悪寒，呼吸困難などである．血圧低下，気道狭窄などのgrade 3以上の反応をきたす症例は3%程度であるが，0.1%の症例で致死的経過をたどることがあり，注意が必要である．抗アレルギー薬の前投薬が推奨されている．

3．Matuzumab(EMD72000)

Matuzumabは，EGFRに対するヒト化IgG1抗体である．すなわち，抗原を認識する部分である相補性決定部位のみマウス由来のタンパクで，それ以外の95%はヒト由来のタンパクでできている抗体である．第Ⅰ相試験では標準治療に不応となったEGFR陽性の大腸がん，食道がん，頭頸部がんの患者22名に投与され，PR 23%，SD 27%，アクネ様皮疹の割合は64%と報告されている[15]．本邦でもEGFR陽性の大腸がん，胃がん，食道がん患者26名に第Ⅰ相試験として投与され，大腸がんにおいて2例のPR, 10例のSD症例を認めている[16]．現在化学療法との併用による胃がんでの検討[17]や，頭頸部がんでの検討[18]が行われている．

4．Panitumumab(ABX-EGF)

PanitumumabはEGFRに対するヒトIgG2抗体である．100%ヒト由来のタンパクでできているため，薬剤に対する抗体ができないのが特徴で，他の抗EGFR抗体よりもより薬物動態プロファイルの改善が期待できる．Panitumumab単剤投与では，標準的治療に不応になった結腸直腸がん患者に対するphase Ⅱにおいて，PR 11%，SD 36%と良好な成績を収めた[19]．皮膚毒性は95%に認められたが，grade 3以上の頻度は7%であった．皮膚毒性以外では，倦怠感51%(grade 3以上9%)，嘔気39%(同3%)，下痢36%(2%)，腹痛30%(3%)，などであった．Cetuximabに認められるinfusion reactionも認められていない．また，2005年11月には，標準的治療に不応となった463名をランダム化して行われたphase Ⅲ試験において，panitumumab単剤投与はbest supportive care群に比べて有意に無増悪生存期間を延長すると発表された．これにより2006年にはアメリカで承認される見込みである．また，非小細

表 2-a BOND study—抗腫瘍効果—

	CPT11＋Cetuximab (n＝218)	Cetuximab (n＝111)	*p*
CR＋PR	23%	11%	0.0074
CR＋PR＋SD	56%	32%	0.0001
MedianTTP	4.1mon	1.5mon	＜0.0001
MST	8.6mon	6.9mon	0.48

表 2-b BOND study—有害事象(grade 3/4)—

	CPT11＋Cetuximab (n＝218)	Cetuximab (n＝111)
下痢	45(21.2%)	2(1.7%)
倦怠感	29(13.7%)	12(10.4%)
アクネ様皮疹	20(9.4%)	6(5.2%)
好中球減少	20(9.5%)	0
嘔気/嘔吐	15(7.1%)	5(4.3%)
貧血	10(4.8%)	3(2.7%)
腹痛	7(3.3%)	6(5.2%)
血小板減少	1(0.5%)	1(0.9%)
過敏症	0	4(3.5%)
Cetuximab関連死	0	0

表 2-c BOND study—皮膚毒性と抗腫瘍効果の関係—

	CPT11＋Cetuximab		Cetuximab	
	RR	MST	RR	MST
皮膚毒性なし	6 %	3.0mon	0 %	2.5mon
皮膚毒性あり	26%	9.1mon	13%	8.1mon

胞肺がん，前立腺がん，腎細胞がんについても臨床第Ⅱ相試験が行われており，併用療法についても検討されている．

5．EGFR発現と抗腫瘍効果

乳がんの治療におけるtratuzumabは，標的分子であるErbB2の発現と抗腫瘍効果がよく相関することが知られている．Cetuximabも当初EGFR陽性大腸がんに対して治療開発が進められてきたが，BOND studyでも示されたように，cetuximabの抗腫瘍効果はEGFRの発現強度とまったく相関を認めなかった．同じくEGFRを標的とするgefitinibも同様に抗腫瘍効果と発現強度が相関しないことが一つの謎であったが，EGFRのATP結合部位(exon 19)の変異が効果予測因子になるとの報告がなされた[20]．大腸がんでも同様の検討がなされたが，大腸がんでのEGFRの変異はほとんど認められなかった．EGFR遺伝子の増幅が抗腫瘍効果と相関するとの報告もある[21]が，さらなる検討が必要と思われる．

VEGF(vascular endothelial growth factor)を標的にした抗体療法

1．がんとVEGF

血管新生は，組織の増殖において，重要な役割を担っていると考えられている．それはがんの増殖でも同様である．血管新生を阻害することで抗腫瘍効果が発揮されるという仮説が，1971年にFolkmanらにより示唆され[22]，がんが増殖するためには必要な酸素や栄養を提供し，不要な代謝物を排除するための血管網の構築が，どのように行われているかについての研究が，この数十年行われてきた．

1980年代，Sengerらは移植腫瘍による腹水貯留の機序の研究において，腫瘍細胞から分泌され血管透過性を更新するポリペプチドの存在を報告し，VPF(vascular permability factor)と命名した．また，Ferraraらは，下垂体の濾胞細胞の培養液から血管内皮細胞の増殖を促進する因子を同定，VEGF(vascular endothelial growth factor)と命名したが，両者は同一の物質であることが後に判明した[23]．

VEGF-Aには，8つのスプライシングバリアントがあることが知られ，それぞれ若干細胞への親和性などが異なるといわれている．大まかな

表 3 大腸がん初回治療例に対するcetuximab＋抗がん剤併用療法phase II の成績

	IFL ＋Cetuximab (n＝29)	CPT11/5FU/LV(AIO) ＋Cetuximab (n＝21)	FOLFIRI ＋Cetuximab (n＝22)	FOLFOX ＋Cetuximab (n＝62)
CR＋PR	48%	67%	46%	81%
SD	41%	29%	41%	17%
PD	11%	5 %	14%	2 %
Ref.	AH. Rosenberg ASCO2002[38]	G. Folprecht ASCO2005[39]	P. Rougier ASCO2004[40]	E. Díaz Rubio ASCO2005[41]