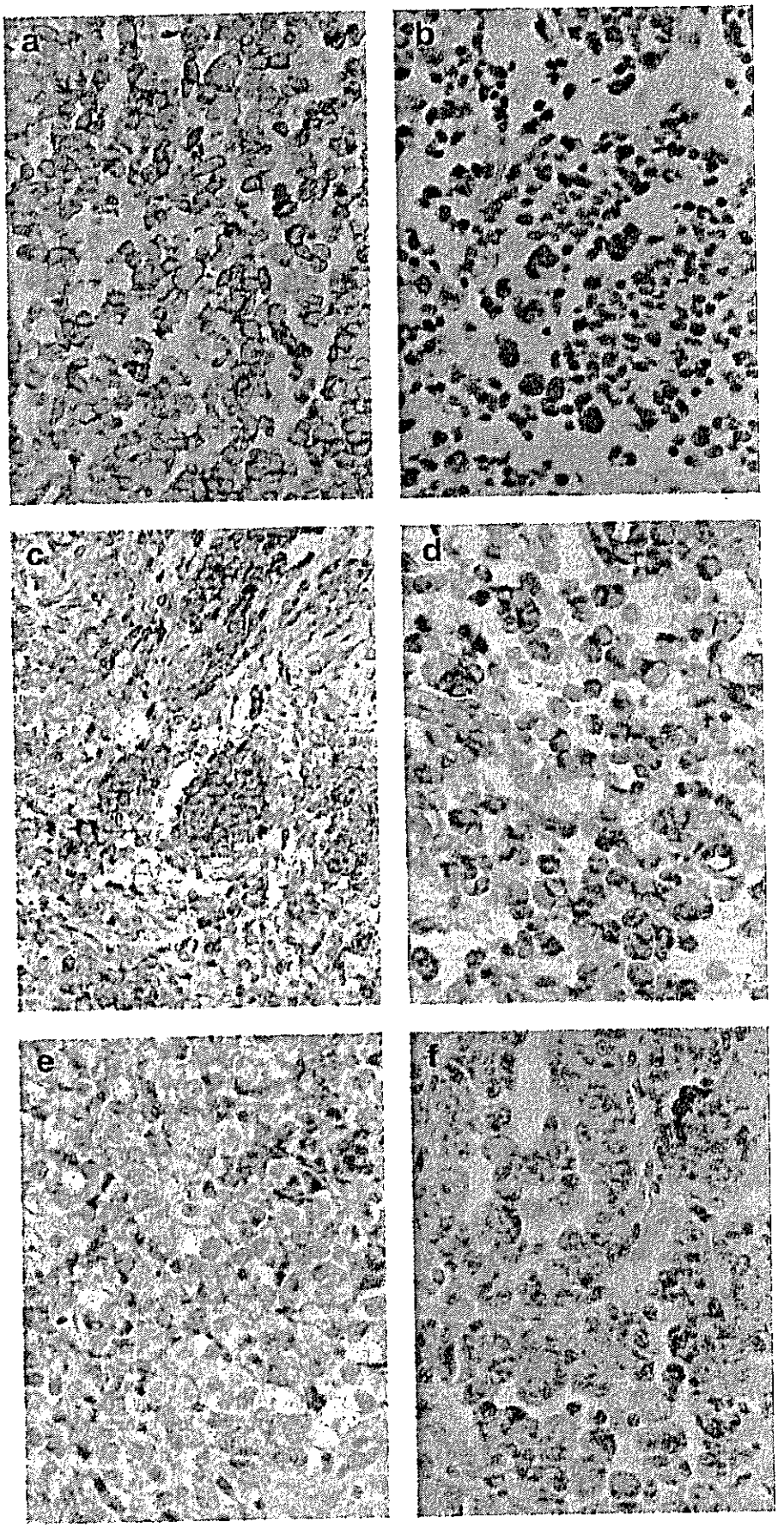

FIGURE 3 – Immunohistochemical expression of ABC transporter protein in control sections and various types of soft tissue sarcoma. (*a*) MNNG/HOS/DXR 1000 cell line for positive control shows strong membranous immunoreaction for P-glycoprotein. (*b*) Its negative control reveals no immunoreactivity. (*c*) Immunohistochemical staining of P-glycoprotein in monophasic fibrous synovial sarcoma arising in the thigh of a 40-year-old male. Tumor cells show membranous positive immunoreactivity. (*d*) Membranous immunostaining for MRP1 in MPNST arising in the spinal canal of a 2-year-old girl. (*e*) MRP2 is localized on the plasma membrane of a clear cell sarcoma in the lower leg of a 19-year-old female. (*f*) MRP3 is localized in the cytoplasm of an alveolar soft part sarcoma in the axillary region of a 16-year-old male.

TABLE IV - CORRELATION BETWEEN REAL-TIME QUANTITATIVE RT-PCR AND IMMUNOHISTOCHEMISTRY

MDR1 mRNA (A.U.)				
		Average	SD	
P-gp: IHC[1]	+ (n=32)	186.96	323.91	p=0.0002*
	− (n=54)	12.72	48.47	
MRP1 mRNA (A.U.)				
		Average	SD	
MRP1: IHC[1]	+ (n=37)	98.96	115.91	p=0.0001*
	− (n=49)	24.31	53.02	
MRP2 mRNA (A.U.)				
		Average	SD	
MRP2: IHC[1]	+ (n=27)	2.04	3.67	p=0.0001*
	− (n=59)	0.13	0.23	
MRP3 mRNA (A.U.)				
		Average	SD	
MRP3: IHC[1]	+ (n=31)	301.42	405.51	p=0.0002*
	− (n=55)	71.12	127.48	

[1]IHC: Immunohistochemistry.–*Statistically significant.

TABLE V - CORRELATION BETWEEN CLINICAL AND PATHOLOGICAL PARAMETERS AND ABC TRANSPORTER PROTEIN EXPRESSION[1]

Parameters	PGP		MRP1		MRP2		MRP3	
	+	−	+	−	+	−	+	−
Age (n=83)								
< 20 yrs. (n=11)	4	7	8	3*	4	7	5	6
≥ 20 yrs. (n=72)	28	44	28	44*	22	50	25	47
Sex (n=83)								
Male (n=44)	16	28	16	28	12	32	16	28
Female (n=39)	16	23	20	19	14	25	14	25
Location (n=83)								
EX and TR (n=63)	26	37	30	33	21	42	22	41
AC or TC, RP or Vis (n=20)	6	14	6	14	5	15	8	12
Size (n=83)								
< 5 cm (n=22)	3	19*	8	14	4	18	7	15
≥ 5 cm (n=61)	29	32*	28	33	22	39	23	38
Depth (n=83)								
Superficial (n=5)	2	3	0	5	2	3	2	3
Deep (n=78)	30	48	36	42	24	54	28	50
Mitosis (n=86)								
0–19/10 HPFs (n=67)	25	42	31	36	25	42*	27	40
≥ 20/10 HPFs (n=19)	7	12	6	13	2	17*	4	15
Necrosis (n=86)								
0–50% (n=78)	29	49	33	45	24	54	30	48
> 50% (n=8)	3	5	4	4	3	5	1	7
AJCC stage (n=83)								
Low (Stage I, II) (n=32)	8	24*	12	20	9	23	11	21
High (Stage III, IV) (n=51)	24	27*	24	27	17	34	19	32

[1]+: positive, −: negative, p: p values.–*Statistically significant difference in immunohistochemical expression between the 2 groups of clinicopathologic parameters, EX: Extremities, TR: Trunk, AC: Abdominal cavity, TC: Thoracic cavity, RP: Retroperitoneum, Vis: Viscera, AJCC: American Joint Committee on Cancer, HPFs: High-power fields.

The MIB-1 LI ranged from 1.3 to 67.6. It demonstrated no correlation with either ABC transporter mRNA expression or with ABC transporter protein.

Discussion

Except extraskeltal Ewing's sarcoma and rhabdomyosarcoma, the value of systemic chemotherapy remains controversial.[23] According to the review of van Glabbeke *et al.*,[28] the responses to chemotherapy in all soft tissue sarcomas are still low (26%). In their series, MPNSTs are not mentioned as separate groups with bad response. Furthermore, Stein *et al.*[29] reported that 7 out of 8 MPNSTs (neurofibrosarcomas) (88%) showed intermediate *MDR1* expression at high or intermediate frequency. In our study, expressions of *MDR1* and *MRP3* in MPNST were significantly higher than those in other sarcomas. Although MDR1/P-gp contributes to drug resistance in a wide spectrum of anti-cancer agents, MRP3 has been reported to confer only low levels of resistance to etoposide and teniposide *in vitro*.[30] On the other hand, Steinbach *et al.*[31] recently demonstrated that a high level of *MRP3* gene expression is correlated with failure of remission in AML and they suggested that this gene expression is involved in drug resistance in childhood AML. Moreover, they also demonstrated a positive correlation between *MRP3* mRNA expression and poor outcome in childhood ALL, especially in the case of the T-cell immunophenotype.[32] Overexpression of these 2 types of ABC transporter protein mRNA may have an important role to play in the poor chemoresponse seen in cases of MPNST. These 2 ABC transporter expressions are possible targets for the development of specific drugs to overcome multidrug resistance in MPNST.

Some investigators have reported that the tumor suppressor protein, wild-type p53, repressed the transcription of the *MDR1* gene *in vitro*,[9–11] while Li *et al.*[33] showed that the induction of wild-type p53 protein activates the expression of the *MDR1* gene. As for clinical samples, Linn *et al.*[12] demonstrated a positive correlation between P-gp and the nuclear accumulation of p53 protein in breast cancer using an immunohistochemical method.

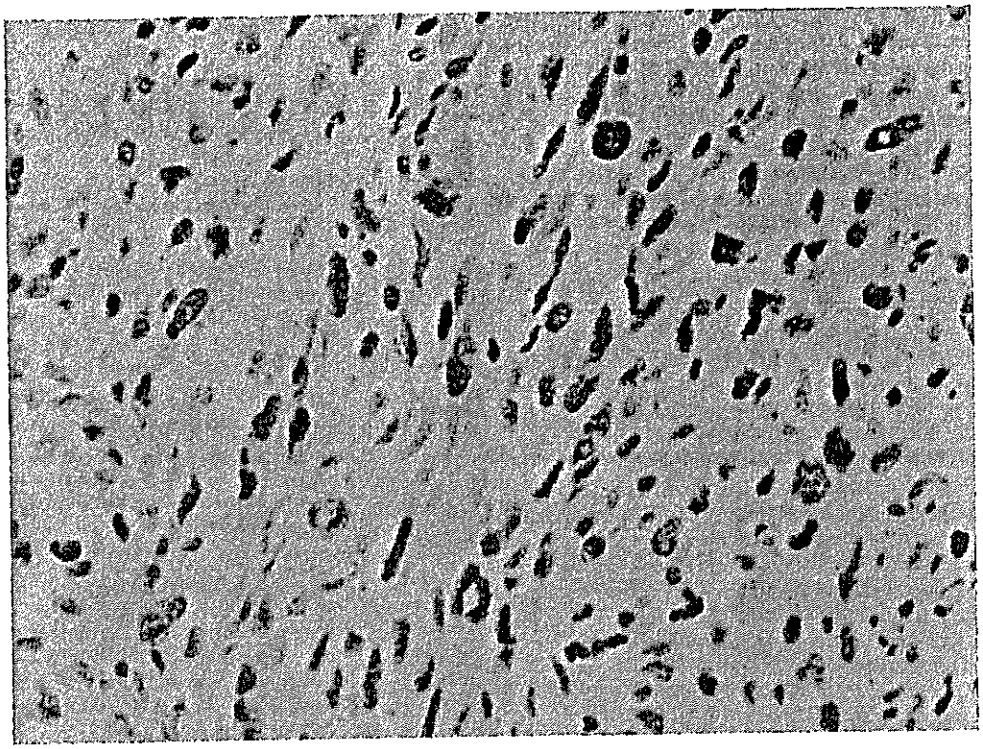

FIGURE 4 – Diffuse nuclear accumulation of p53 protein is observed in pleomorphic MFH arising in the thigh of 76-year-old female.

TABLE VI – CORRELATION BETWEEN ABC TRANSPORTER mRNA EXPRESSION AND IMMUNOHISTOCHEMICAL p53 EXPRESSION

MDR1 mRNA (A.U.)		Average	SD	
p53	+ (n=22)	162.17	382.86	p=0.0328*
p53	− (n=64)	48.47	105.17	
MRP1 mRNA (A.U.)		Average	SD	
p53	+ (n=22)	47.51	43.45	p=0.6054
p53	− (n=64)	59.49	104.96	
MRP2 mRNA (A.U.)		Average	SD	
p53	+ (n=22)	0.55	0.97	p=0.6557
p53	− (n=64)	0.79	2.52	
MRP3 mRNA (A.U.)		Average	SD	
p53	+ (n=22)	248.23	471.39	p=0.0715
p53	− (n=64)	121.79	175.21	

*Statistically significant.

Similar immunohistochemical results have been reported in gastric cancer[13] and oral squamous cell carcinoma.[14] A significant correlation between p53 mutation and *MDR1* mRNA expression has also been documented in advanced colon cancer[34] and in nonsmall cell lung cancer.[35] Paradoxically, a negative correlation between p53 expression and *MDR1* mRNA was also reported in colon cancer,[36] myelodysplastic syndrome[37] and CLL.[38] In our study, aberrant nuclear accumulation of p53 was found to be significantly correlated with a high level of *MDR1* mRNA in soft tissue sarcomas, although the clone of p53 antibody (PAb 1801) was different from that used in the study of Linn *et al.*[12] (DO1). As for the transcriptional regulation of *MRP1*, wild-type p53 has also been shown to repress the transcription of the human *MRP1* promoter.[15–17] However, the immunohistochemical nuclear accumulation of p53 protein has been found to be correlated with MRP1 expression in clinical nonsmall lung cancer,[18] gastric cancer[19] and colon cancer.[20] In this study, no significant correlation was observed between p53 expression and *MRP1* mRNA expression. Stein *et al.*[39] reported that hyperthermia causes nuclear translocation of the Y-box transcription factor, YB-1, and that it is associated with *MDR1* and *MRP1* gene activity in human colon carcinoma cells. Recently, we demonstrated that nuclear localization of YB1 protein is correlated with the overexpression of P-gp and *MDR1* mRNA in synovial sarcoma.[21]. Further studies are required to assess the correlation between nuclear YB-1 expression and the ABC transporter gene expression within several kinds of soft tissue sarcoma.

MDR1 mRNA expression in soft tissue sarcomas has been reported by some investigators using Northern blotting,[40] dot blot,[41] or RT-PCR assay.[42] No authors have analyzed several kinds of ABC transporter mRNA expressions systematically in soft tissue sarcomas. We previously failed to reveal relationship between *MDR1* expression by RNA level with simple RT-PCR method and the protein level with immunohistochemistry.[42] In our study, we showed significant relationship between the different immunohistochemistry and real-time RT-PCR technique to demonstrate all ABC transporter expression in several kinds of soft tissue sarcomas.

It remains unclear as to whether *MDR1*/P-gp or some other ABC transporter expression could be associated with tumor progression or could be used as a marker for chemosensitivity in soft tissue sarcomas. In colon cancer, high level of P-gp expression has been reported to correlate with local tumor aggressiveness such as vessel invasion and lymph node metastases,[43] and disease relapse.[44] Chan *et al.*[45] demonstrated that increased P-gp is associated with poor prognosis but that it is not correlated with chemotherapy response in pediatric soft tissue sarcomas. Nakanishi *et al.*[46] also documented that soft tissue tumors expressing P-gp had a less favorable prognosis than P-gp-negative tumors in both high- and intermediate-grade tumors. They also showed a significantly frequent P-gp positivity in high-grade sarcomas, compared to intermediate- or low-grade sarcomas, according to the AgNOR counting grading system. Moreover, Jimenez *et al.*[47] showed that P-gp-positive high-grade soft tissue sarcomas demonstrate a poor pathologic response to chemotherapy. On the other hand, Coley *et al.*[48] documented that there was no correlation between P-gp expression and prognosis, while Komdeur *et al.*[49] showed that MDR status including P-gp and MRP1 had no significant correlation with tumor response in soft tissue sarcoma. They also showed downregulation of P-gp during the metastatic progression in soft tissue sarcoma.[50] Moreover, They[51] recently demonstrated that immunohistochemical expression of MRP1 and lung resistance-related protein (LRP) significantly correlated with tumor grade in soft tissue sarcomas. In our study, we examined the expressions of MDR1/P-gp, MRP1, MRP2 and MRP3 by both immunohistochemistry and quantitative RT-PCR methods in a large series of soft tissue sarcomas and we found a significant correlation between P-gp expression and large-sized tumors (≥5 cm) or high AJCC staged tumors (stage III and IV), factors which have generally been considered to be adverse prognostic factors in soft tissue sarcomas,[52] although our patients group had received no preoperative chemotherapy. Large tumor size and high AJCC stage also tended to reveal a high level of *MDR1* mRNA in our series, although the difference was not statistically significant.

Breast cancer resistance protein (BCRP) is a newly discovered ABC transporter isolated from breast cancer cells.[53] Unfortunately, we could not analyze BCRP expression in our study; however, its expression should be involved in the future study because BCRP also confers resistance to a variety of anti-cancer drugs.

In conclusion, we showed the relationship between the different 2 techniques used to demonstrate ABC transporters in soft tissue sarcomas. Our results suggest that MDR1/P-gp expression may have an important role to play in tumor progression in cases soft tissue sarcoma, and p53 protein could be 1 of the transcriptional regulators of the *MDR1* gene.

Acknowledegments

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HER2 Overexpression Increases Sensitivity to Gefitinib, an Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor, through Inhibition of HER2/HER3 Heterodimer Formation in Lung Cancer Cells

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Abstract

Gefitinib (Iressa), an epidermal growth factor receptor targeting drug, has been clinically useful for the treatment of patients with non–small cell lung cancer (NSCLC). Gefitinib is currently being applied in clinical studies as either a monotherapy, or as part of a combination therapy against prostate, head and neck, gastric, breast, and colorectal tumors. However, success rates vary between different tumor types, and thus it is important to understand which molecular target(s) are responsible for limiting the therapeutic efficacy of the drug. In this study, we ask whether expression of HER2 affects sensitivity to gefitinib in human lung cancer cells. We established two clones, LK2/HER2-32 and LK2/HER2-57, by transfecting HER2 cDNA into LK2, a NSCLC line with a low expression level of HER2. We observed no mutations in exons 18, 19, and 21 of EGFR gene in LK2, LK2/mock- and two HER2-trasfectants when we observed in-frame deletion mutations (E746-A750) adjacent to K745 in a gefitinib-sensitive NSCLC cell line, PC9. These LK2/HER2-32 and LK2/HER2-57 were much more sensitive to the cytotoxic effects of gefitinib than the parental LK2 lines. Treatment with 0.5 to 1 μmol/L gefitinib specifically blocked Akt activation in both HER2-transfectant lines, but not in the parental LK2 cells. Extracellular signal-regulated kinase-1/2 activation, however, was not blocked by gefitinib up to 10 μmol/L in either the parent or transfectant lines. Gefitinib was also shown to induce cell cycle arrest in the G_1-S phase, and an accompanying increase of $p27^{Kip1}$ was observed. LK2/HER2 transfectants showed constitutive formation of HER2/HER3 heterodimer, which were seen to associate with a regulatory subunit of phosphoinositide-3-kinase, p85α, when active. Treatment of LK2/HER2 cells with gefitinib markedly decreased the formation of HER2/HER3 heterodimers, HER3 basal phosphorylation, and the association of p85α with HER3. This study is the first to show that under basal growth conditions, HER2 sensitizes low-EGFR NSCLC cell lines to growth inhibition by gefitinib. (Cancer Res 2005; 65(10): 4253-60)

Introduction

The epidermal growth factor receptor (EGFR) family (EGFR/HER1/erbB1, HER2/neu, HER3/erbB3, and HER4/erbB4) operates a complex signal transduction cascade through phosphoinositide-3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK)1/2, and modulates proliferation, migration, adhesion, differentiation, angiogenesis, and apoptosis of cancer cells (1–3). Overexpression of EGFR is often associated with advanced tumor stages, metastasis, and poor clinical outcome of several human malignant tumors such as non–small cell lung cancer (NSCLC), and cancers of breast, cervix, head and neck, esophagus, and colorectum (4–7). During the last decade, the EGFR family has been targeted in order to develop novel anticancer drugs in the form of small molecules or monoclonal antibodies (8–10).

Gefitinib (Iressa, ZD1839) is an orally active, selective EGFR tyrosine kinase inhibitor that blocks the signal transduction pathway implicated in cancer growth (11, 12). It has antiproliferative activity in various human cancer cell types *in vivo* as well as *in vitro* (13, 14). Clinically significant antitumor activity was observed in two phase II trials of gefitinib monotherapy in pretreated patients with advanced NSCLC (IDEAL 1 and 2), and gefitinib is now approved in several countries including Japan, Australia, and the U.S. for the treatment of advanced NSCLC (15, 16). Extensive clinical testing, using gefitinib as a monotherapy or as part of a combination therapy, has enabled a number of treatments to be developed against prostate, head and neck, gastric, breast, and colorectal cancers (17). Gefitinib is a promising agent for the treatment of a wide range of tumor types and shows improved therapeutic efficacy when combined with radiation or chemotherapy in various cell lines and xenografts (13, 14, 18–20).

A recent highlight is the novel finding of intrinsic importance that a subgroup of patients with NSCLC has specific mutations in the EGFR genes are well correlated with clinical responsiveness to gefitinib (21). These EGFR mutations lead to increased growth factor signaling and confer susceptibility to gefitinib. Another relevant study by Paez et al. (22) has also reported that somatic mutations of the EGFR gene in 15 of 58 unselected NSCLC tumors from Japan and 1 of 61 from the U.S., and treatment with gefitinib was found to cause tumor regression in NSCLC patients more frequently in Japan than in the U.S. Screening for such mutations in the EGFR gene in NSCLC may identify patients who will have a responsiveness to gefitinib (21, 23). Moreover, we have recently observed that susceptibility to gefitinib is closely correlated with EGFR-dependent Akt and ERK1/2 activation levels among nine NSCLC cell lines under basal growth conditions (23). Consistent with this study (23), treatment with a

Note: "Iressa" is a trademark of the AstraZeneca group of companies.

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very low dose of gefitinib of the drug-sensitive NSCLC cell line with EGFR mutations resulted in almost complete inhibition of EGFR autophosphorylation and the phosphorylation of its downstream targets, ERK1/2 and Akt (22).

On the other hand, overexpression of HER2 in various cancer cell lines or xenografts increases cytotoxicity and/or the antitumor effects of gefitinib (24–27). HER2/erbB2, a 185 kDa transmembrane protein tyrosine kinase, is overexpressed by gene amplification and constitutively activates cancer cell proliferation (28). Amplification of the HER2 gene has been reported in several types of cancer including breast, ovarian, stomach (29, 30), and NSCLC (31, 32), and most of these cases have been associated with poor prognosis (33–35). Of the HER family proteins, gefitinib inhibits EGFR/HER1 phosphorylation with an IC_{50} of 27 to 33 nmol/L and HER2 phosphorylation with an IC_{50} of 3.7 μmol/L, suggesting about 100-fold difference in the drug sensitivity for EGFR and HER2 (11). HER3 is deficient in the tyrosine kinase domain and therefore shows no affinity to gefitinib. Because no studies seem to have addressed how HER2 overexpression modulates sensitivity to gefitinib in human cancer cells, we established isogenic cell lines with or without HER2 overexpression, in order to determine the mechanism of HER2 action. We have recently observed that one NSCLC cell line, LK2, expresses little, if any, EGFR and HER2, but moderately expresses HER3 (23). In our present study, we have established two sublines of LK2 (LK2/HER2) that have been transfected with wild-type HER2 cDNA, and we have examined how drug sensitivity to gefitinib is differentially controlled between LK2 and LK2/HER2.

Materials and Methods

Materials. The following materials were obtained from the indicated sources: Gefitinib (AstraZeneca, Macclesfield, United Kingdom); anti-HER2, anti-HER3, anti-p85α, and anti-phospho-HER2 antibodies (Upstate Biotechnology, Lake Placid, NY); antibodies to ERK1/2, phospho-ERK1/2, Akt, phospho-Akt and $p21^{WAF1/CIP1}$ (Cell Signaling Technology, Beverly, MA); anti-P-Tyr antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-β-actin antibody, propidium iodide and RNase A (Sigma, St. Louis, MO); anti $p27^{Kip1}$ antibody (BD Transduction Laboratories, San Jose, CA); anti-HER3 antibody for immunoprecipitation (NeoMarkers, Montreal, Quebec, Canada).

Cell culture. LK2 cells were purchased from Japanese Collection of Research Bioresources (Tokyo, Japan). PC9 and QG56 cells were kindly provided from Dr. Yukito Ichinose (Kyushu Cancer Center, Fukuoka, Japan). These cells were cultured in RPMI supplemented with 10% fetal bovine serum (23). LK2/HER2-32 and LK2/HER2-57 cells were established after stable transfection with pIREShyg2 expression plasmids (Clontech Laboratories, Palo Alto, CA) using Lipofectin 2000 Reagent (Invitrogen, San Diego, CA). These cells were cultured in RPMI supplemented with 10% fetal bovine serum and 350 μg/mL hygromycin, and were maintained under standard cell culture conditions at 37°C and 5% CO_2 in a humid environment.

Cell growth assay. Cell growth curves were determined by plating 5×10^3 cells in a 24-well plate. After 24 hours, 20 ng/mL of EGF or vehicle was added, followed by incubation at 37°C. The medium was replaced every other day with fresh medium containing either EGF or vehicle, and cells were counted by a Coulter counter at indicated times.

Western blot and immunoprecipitation. Subconfluent tumor cells cultured in medium supplemented with 10% fetal bovine serum were incubated with various concentrations of gefitinib for 3 hours at 37°C. The cells were then rinsed with ice-cold PBS and lysed in Triton X-100 buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1% Triton X-100, and 10% glycerol containing 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L sodium vanadate), and cell lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA) as described previously (23, 36). After transfer, blots were incubated with the blocking solution and probed with anti-HER2 antibody, anti-HER3 antibody, anti-p85α antibody, anti-β-actin antibody, anti-phospho-HER2 antibody, anti-ERK1/2 antibody, anti-phospho-ERK1/2 antibody, anti-Akt antibody, anti-phospho-Akt antibody, anti-p21 antibody, or anti-p27 antibody followed by washing. The protein content was visualized using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham, Piscataway, NJ). For immunoprecipitation, 4 mg of total protein from cell lysates using NP40 buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 80 mmol/L NaCl, 0.3% NP40, and 10% glycerol containing 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L sodium vanadate) was incubated for 2 hours with anti-HER3 antibody and Protein A/G plus-agarose (Santa Cruz Biotechnology) and gently shaken. The precipitates were washed thrice with ice-cold lysis buffer and resolved by SDS-PAGE followed by Western blot analysis.

Cell viability assay. CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Madison, WI) was used to evaluate cytotoxicity in LK2 stable transfectants. One hundred microliters of an exponentially growing cell suspension ($3\text{-}5 \times 10^3$ cells) was seeded into a 96-well plate. The following day, various concentrations of gefitinib were added. After incubation for 72 hours at 37°C, 100 μL of CellTiter-Glo Reagent were added to each well and the plates were shaken gently for 2 minutes. After incubation for 10 minutes at room temperature, luminescence was measured using a multilabel counter (Wallac, Tokyo, Japan) (23, 36). Each experiment was done using three replicate wells for each drug concentration.

Colony formation assay. Cell survival was determined by plating 3 to 9 $\times 10^2$ cells in 35-mm dishes. After 24 hours, various concentrations of gefitinib were added, followed by incubation for 7 to 10 days at 37°C. Gefitinib was solubilized in DMSO. Control experiments were done by adding equivalent volumes of DMSO to plates. Colonies were counted after Giemsa staining as described previously (23).

Fluorescence-activated cell sorting analysis. The cells were seeded in a 6-cm dish and allowed to grow overnight. Gefitinib (1 or 5 μmol/L) was added for 48 hours at 37°C and cells were then and fixed overnight in ethanol at 4°C. Fixed cells were resuspended in a propidium iodide solution (15 μg/mL) containing RNase A (50 μg/mL) before incubation at room temperature for 1 hour. Cell cycle analysis was done using FACScan and Cell Quest software (Becton Dickinson Labware, Mountain View, CA).

Results

Overexpression of HER2 increases sensitivity to gefitinib in LK2 cells. First, we asked whether HER2 overexpression affects sensitivity to gefitinib by examining LK2/parent cells, LK2/mock cells, and HER2 cDNA transfectants. Two HER2 transfectants (LK2/HER2-32 and LK2/HER2-57) were isolated after introduction of human HER2 cDNA into LK2 cells expressing moderate levels of HER3, and very low levels of EGFR and HER2. These HER2 transfectants showed much higher HER2 expression than their parental counterparts, LK2 cells, and LK2/mock cells (Fig. 1*A*). We observed no apparent differences in the growth rates between LK2/mock and its two HER2 transfectants under exponential growth conditions in the absence or presence of EGF (Fig. 1*B*).

The expression levels of HER3 were similar among the four cell lines. Both LK2/HER2-32 and LK2/HER2-57 cells showed an approximately 2-fold greater sensitivity to the cytotoxic effects of gefitinib than their parental counterparts, when IC_{50} values were determined by cell survival assay (Fig. 2*A*). Both HER2 transfectants showed similar sensitivities to gefitinib when assayed by cell survival assay. A separate assay, which was assessed by colony formation, showed LK2/HER2-57 cells to be approximately 5-fold more sensitive to gefitinib than LK2/mock cells and QG56 cells (Fig. 2*B*). However, sensitivity was about 20-fold lower in LK2/HER2-57 cells than in PC9 cells, which harbor in-frame deletion mutation of EGFR (E746-A750) in exon 19 (23). Our previous

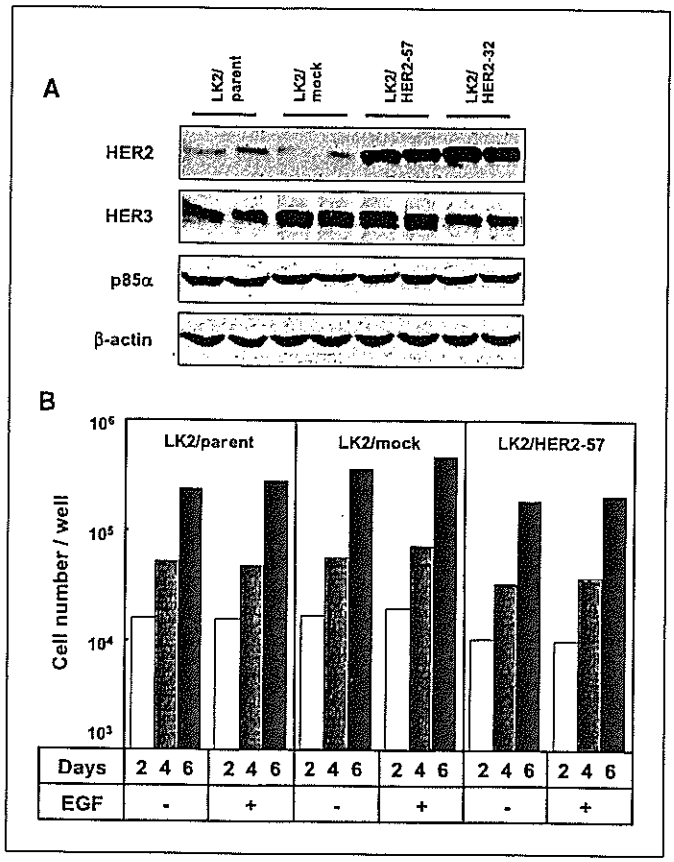


Figure 1. Overexpression of HER2 does not alter growth rate with or without EGF. *A*, comparison of HER2, HER3, and p85α protein expression levels in LK2, LK2/mock, and its two HER2 transfectants. Protein extracts were resolved by 7.5% SDS-PAGE and probed with each antibody. Immunoreactive proteins were visualized by enhanced chemiluminescence. *B*, cell growth rates are evaluated in LK2 and its HER2 transfectants under normal growth conditions with or without 20 ng/mL of EGF. Cells were harvested by trypsinization and counted by a Coulter counter at the indicated time intervals after seeding. Columns, mean value of three dishes, and each value is within the range of 10% error.

research has shown that among nine NSCLC cell lines tested, PC9 is the most sensitive to gefitinib, and QG56 and LK2 are the most resistant (23). Thus, overexpression of HER2 seems to alter the sensitivity of LK2 cells to gefitinib.

The presence or absence of EGFR mutations plays a key role in the drug sensitivity of NSCLC cells to gefitinib (21, 22). We examined the absence or presence of any mutation in exons 18, 19, and 21 of EGFR gene, and found no mutation in LK2, LK2/mock, two HER2 transfectants and QG56 (data not shown).

Gefitinib inhibits Akt phosphorylation in a dose-dependent manner in LK2/HER2 cells. HER2 activates a number of cytoplasmic signal transduction pathways including the PI3K/Akt pathway and the Ras/MAP kinase pathway. We compared the effects of gefitinib on phosphorylation of HER2, Akt, and ERK1/2 in four cell lines: LK2/parent, LK2/mock, LK2/HER2-32, and LK2/HER-57. Figure 3*A* shows the effects of gefitinib on phosphorylation of HER2, Akt, and ERK1/2 in these cell lines under basal growth conditions in the presence of 10% serum. In all four cell lines, HER2, Akt, and ERK1/2 were phosphorylated in the absence of the drug, although HER2 phosphorylation levels in LK2/parent and LK2/mock cells were much lower than that in LK2/HER2 cell lines. P-Akt was found to be 1.9- and 1.7-fold higher in LK2/HER2-32 and LK2/HER2-57, respectively, than in LK2/mock in the absence of drug (Fig. 3*A*). Moreover, P-ERK1/2 in both LK2/HER2-32 and LK2/HER2-57 was about 1.3-fold higher than that in LK2/mock in the absence of drug.

Figure 3*B* shows inhibition dose kinetics of gefitinib for Akt and ERK1/2 in these cells, with the phosphorylation activity of these targets without gefitinib normalized as 100%. In LK2/parent cells and LK2/mock cells, activation of both Akt and ERK1/2 was not changed by gefitinib up to 10 μmol/L (Fig. 3*A* and *B*). However, in the two LK2/HER2 cell lines, Akt activation was inhibited by gefitinib in a dose-dependent manner, being blocked at 50% control on application of 1 μmol/L gefitinib. Application of 5 to 10 μmol/L gefitinib reduced HER2 phosphorylation by 40% to 60% of the activity in the absence of drug, whereas gefitinib did not show any inhibitory effect on ERK1/2 activation (Fig. 3*A* and *B*). Overexpression of HER2 thus enhanced drug sensitivity to gefitinib in LK2 cells and also specifically sensitized the gefitinib-induced inhibition of PI3K/Akt pathway.

Gefitinib arrests the cell cycle at G_1 and increases $p27^{Kip1}$ expression levels in LK2/HER2 cells. To examine whether the inhibitory effects observed in cell growth assays reflect a delay or arrest of cell cycle in the G_0/G_1 phase, as shown previously (37–39), cells were treated with gefitinib for 48 hours, and cell cycle progression was evaluated by fluorescence-activated cell sorting

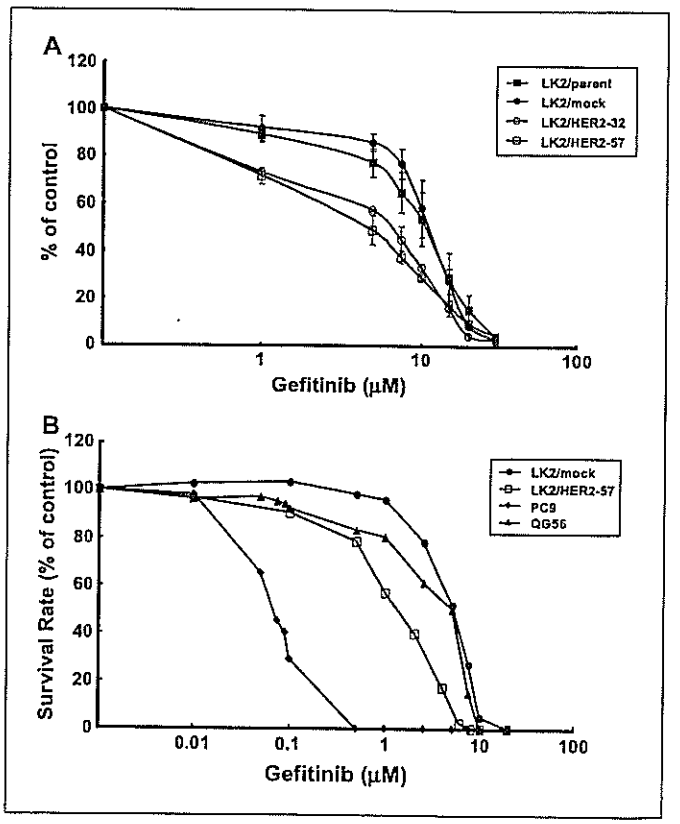


Figure 2. Overexpression of HER2 increases sensitivity to gefitinib in LK2. *A*, dose-response curves of the parental LK2, LK2/mock, and two HER2 transfectants. Drug sensitivity was determined using a cell viability assay in the absence or presence of different doses of gefitinib. The number of viable cells was calculated at 72 hours and graphed as the percentage of untreated cells. Each value is the average of triplicate dishes (SD). *B*, dose-response curves of two NSCLC cell lines (PC9, gefitinib-sensitive cell line; and QG56, gefitinib-insensitive cell line), LK2/HER2 and LK2/mock. Cell survival was determined by colony formation assay in the absence or presence of various doses of gefitinib. The number of colonies after incubation for 7 days with or without gefitinib was presented as a percentage of untreated cells.

analysis. Treatment with gefitinib increased the portion of cells in G_0/G_1 phase by 7% to 8% in the two LK2/HER2 cell lines, with a corresponding decrease in the portion of cells in S and G_2-M phases (Fig. 4*A* and *B*). In contrast, in LK2/mock cells, no change in cell cycle distribution was detected upon treatment with gefitinib. Furthermore, no sub-G_1 fraction, indicative of apoptosis, was observed following gefitinib treatment in any of the cell lines. We subsequently examined the effects of gefitinib on $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ expression, because both of them have been implicated in the growth arrest after disruption of EGFR tyrosine kinase activity in EGFR or HER2 overexpressing cells (refs. 40, 41; Fig. 4*C*). In all cell lines, only a small amount of $p27^{Kip1}$ was expressed in the absence of the drug. Treatment with gefitinib (5 μmol/L) led to an increase in $p27^{Kip1}$ levels: approximately 8-fold in LK2/HER2-32 cells, and 4- to 5-fold in LK2/HER2-57 cells, when there was only a slight if any change in the $p27^{Kip1}$ levels in LK2/mock cells. In contrast, $p21^{WAF1/CIP1}$ expression was unchanged in all of the clones after gefitinib treatment. Although inhibition by gefitinib in both growth curves and Akt activation was similar between LK2/HER2-32 and LK2/HER2-57, a slight but reproducible difference in an increase of $p27^{kip1}$ was observed between the two gefitinib-treated transfectants. However, the underlying mechanism of why this difference in $p27^{kip1}$ expression levels by gefitinib appears remains to be further studied.

Gefitinib inhibits constitutive association of HER3 with HER2 as well as p85α, and basal HER3 phosphorylation in LK2/HER2 cells. LK2 cells expressed no detectable levels of EGFR, but sufficient levels of HER3 (23). HER3 efficiently recruits p85α, but HER2 lacks the appropriate binding site(s) for this (42, 43). Moreover, the constitutive association of HER2/HER3 is often observed in tumor cells overexpressing HER2 (44). We examined the effect of gefitinib on the association of HER2 with HER3, and that of HER3 with p85α. LK2/HER2 and LK2/mock cells were treated with gefitinib for 3 hours and then cell lysates were immunoprecipitated with anti-HER3 antibodies, followed by Western blot analysis (Fig. 5). HER3 was coprecipitated with HER2 in LK2/HER2-32 and LK2/HER2-57 cells under basal growth conditions, indicating the presence of constitutive HER2/HER3 complexes. In LK2/mock cells, no HER2/HER3 heterodimer formation was apparent and HER3 exhibited only slight, if any, tyrosine phosphorylation and almost no

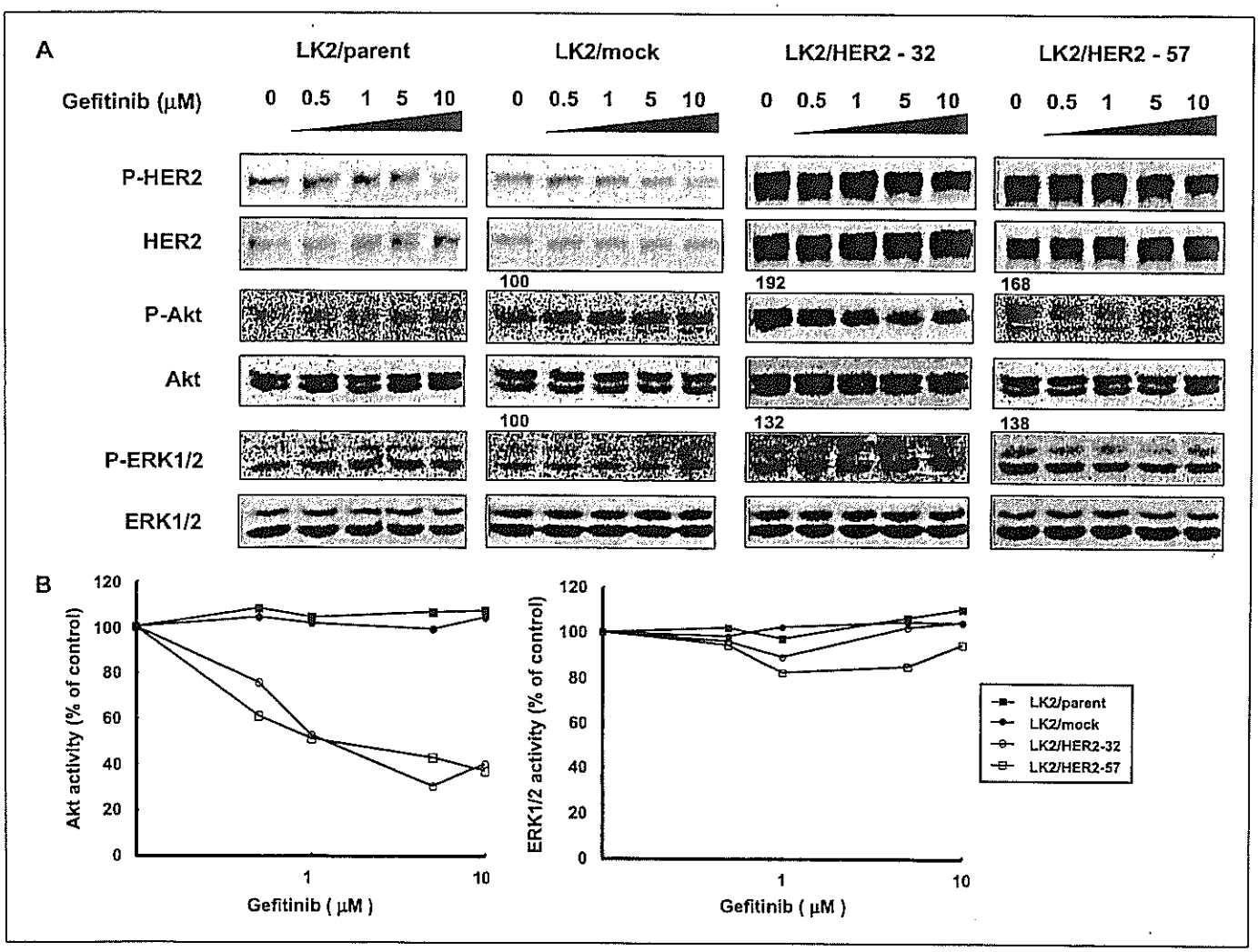
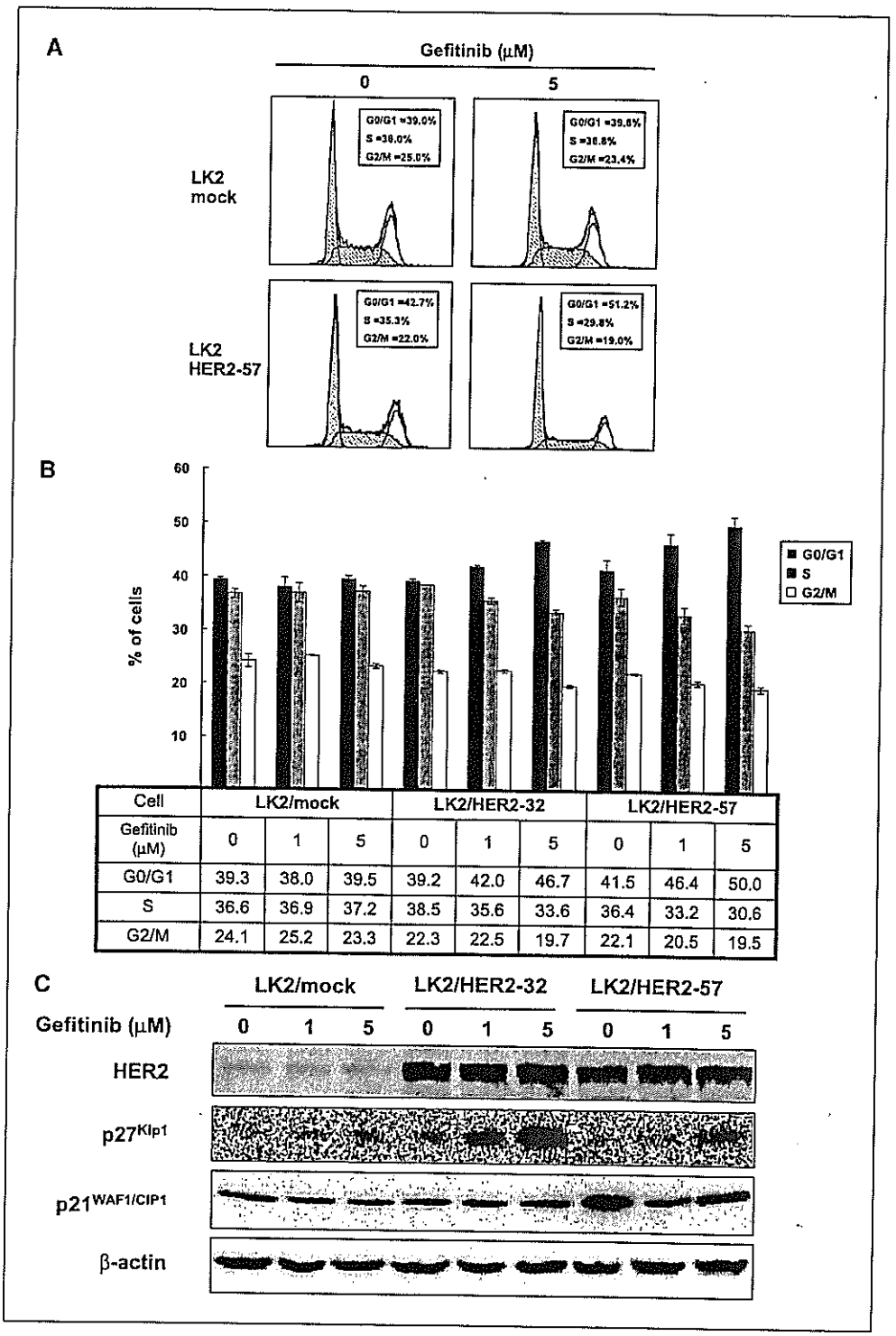


Figure 3. Gefitinib inhibits Akt phosphorylation in a dose-dependent manner in LK2/HER2. *A*, exponentially growing cells in 10% serum medium were pretreated for 3 hours with the indicated concentrations of gefitinib. Protein extracts were resolved by 7.5% SDS-PAGE and probed with antibodies. HER2, Akt, and ERK1/2 activity was determined using each corresponding anti-phospho antibody. Relative levels of P-Akt and P-ERK1/2 in LK2/HER2-32 and LK2/HER2-57 are presented when basal phosphoprotein levels of each Akt and ERK1/2 in LK2/mock is presented as 100%. *B*, quantitative analysis of Akt and ERK1/2 activity was done on the immunoblots using Image Gauge V3.45 software and plotted for each cell line using the 0 μmol/L arm as a control.



Cell	LK2/mock			LK2/HER2-32			LK2/HER2-57		
Gefitinib (μM)	0	1	5	0	1	5	0	1	5
G0/G1	39.3	38.0	39.5	39.2	42.0	46.7	41.5	46.4	50.0
S	36.6	36.9	37.2	38.5	35.6	33.6	36.4	33.2	30.6
G2/M	24.1	25.2	23.3	22.3	22.5	19.7	22.1	20.5	19.5

Figure 4. Gefitinib increases the proportion of cells in the G_0/G_1 phase of the cell cycle and increases $p27^{Kip1}$ expression levels in LK2/HER2. *A*, cell cycle profiles of LK2/HER2 and LK2/mock at 48 hours after treatment with or without gefitinib are presented. Cell cycle distribution was determined by curve-fitting using the ModFit LT software. *B*, the result of cell cycle analysis of LK2/HER2 and LK2/mock. The mean values (SD) for each phase of the cell cycle are shown on the graph and in the table. *C*, exponentially growing cells were pretreated for 48 hours with the indicated concentrations of gefitinib. Protein extracts were resolved by 7.5% SDS-PAGE and probed with either antibody.

association with p85α. In contrast, in LK2/HER2 cell lines, HER3 exhibited a high level of basal tyrosine phosphorylation and association with p85α. Treatment with gefitinib markedly abrogated HER3 phosphorylation as well as the association of HER3 with HER2, and HER3 with p85α.

Discussion

In a recent study, we examined the molecular basis of sensitivity to gefitinib using nine NSCLC cell lines, and we found that PC9 was most sensitive to the drug. These nine cell lines expressed different levels of EGFR, HER2, HER3, and HER4, but there appeared to be no correlation between EGFR and/or HER2 expression and drug sensitivity (23). However, the activation of EGFR, Akt and ERK1/2 was inhibited by much lower concentrations of gefitinib in PC9 cells than in the other eight cell lines under basal growth conditions. This suggests that sensitivity to growth inhibition by gefinitib in NSCLC cell lines is dependent upon the activation levels of Akt and ERK1/2 in response to EGFR signaling for survival and

proliferation (23). Moreover, two established EGFR cDNA transfectants (LK2/EGFR-2 and LK2/EGFR-5) were found to show a similar level of sensitivity to gefitinib in the parental strain (23). In this study, overexpression of HER2 in LK2 cells is shown to result in enhanced drug sensitivity to gefitinib (Fig. 2*A* and *B*). Thus, the expression of HER2, but not EGFR, seems to modulate drug sensitivity to gefitinib in this NSCLC cell line. Therapeutic efficacy of gefitinib against NSCLC was found to be closely associated with somatic mutations in the EGFR kinase domain (21, 22), and we found that one highly gefitinib-sensitive NSCLC cell line, PC9, also has a deletion mutation (E746-A750) in exon 19 of EGFR catalytic domain. LK2, LK2/mock, and its two HER2 transfectants, had no such mutations in exon 18, 19, and 21 of the EGFR catalytic domain, suggesting that HER2 overexpression did not induce EGFR mutation in LK2 cells.

In HER2-transfected LK2 cells under basal growth conditions, application of 0.5 to 1 μmol/L gefitinib specifically inhibited Akt activation, but not ERK1/2 activation. Treatment with gefitinib induced G_0/G_1 arrest as well as the accumulation of $p27^{kip1}$ but not of $p21^{WAF1/CIP1}$. The expression of CDK inhibitors, $p27^{Kip1}$ and $p21^{WAF1/CIP1}$, inhibits formation of the cyclin-CDK complexes essential for G_1 to S phase progression (45). Inhibition of the EGFR-mediated pathway often induces up-regulation of both $p27^{Kip1}$ and $p21^{WAF1/CIP1}$ (40, 41). In our present study, treatment with gefitinib of two HER2 transfectants resulted in no increase in $p21^{WAF1/CIP1}$ expression but in up-regulation of $p27^{Kip1}$ consistent with recent study by Kalish et al. (46). They have shown the down-regulation of cyclin D1 expression by gefitinib with concomitant accumulation of $p27^{Kip1}$, but no change in the expression level of $p21^{WAF1/CIP1}$. Accumulation of $p27^{Kip1}$ protein rather than $p21^{WAF1/CIP1}$ protein seems to be specifically associated with growth arrest by gefitinib in LK2/HER2 transfectants.

Constitutive formation of HER2/HER3 heterodimers, and association of p85α with HER3 were observed, and gefitinib at 1 to 5 μmol/L markedly abrogated these associations. Taken together, overexpression of HER2 seems to sensitize LK2 cells to gefitinib, plausibly coordinated with HER3. A relevant study by Campiglio et al. (47) examined the effect of gefitinib on proliferation and survival, and its activation of Akt and ERK1/2, in six human breast cancer cell lines expressing various levels of EGFR and HER2. This study reported that the effects of gefitinib are independent of EGFR expression levels, but are influenced by high HER2 expression (47). Moreover, recent studies have also reported that gefitinib has a good antitumor effect on tumors displaying higher HER2 levels (24–26). These studies, including our present study, consistently suggest that high HER2 expression confers increased sensitivity to the therapeutic effect of gefitinib. Gefitinib intrinsically shows very high affinity against EGFR, but about 100-fold less affinity against HER2 than EGFR (11). IC_{50} of gefitinib for EGFR phosphorylation was about 0.1 to 0.5 μmol/L in various NSCLC lines (23), whereas IC_{50} of gefitinib for HER2 phosphorylation in LK2/HER2-32 and LK2/HER2-57 were about 10 μmol/L (Fig. 3). In contrast, gefitinib inhibited HER3 phosphorylation in the heterodimer HER2/HER3 at IC_{50} of about 1 μmol/L (Fig. 5), suggesting that HER3 is activated in the heterodimer with HER2, resulting in increased drug sensitivity to the inhibitory effect of gefitinib.

Overexpression of HER2 in LK2 cells resulted in a marked inhibitory effect of gefitinib on Akt phosphorylation, but not on ERK1/2 phosphorylation. HER2 phosphorylation under exponential growth conditions was moderately affected when gefitinib was used at high concentrations. Ras/ERK1/2 signaling is associated with cell proliferation, and Akt signaling is associated with cell survival (3, 48). Thus, HER2 seems to operate through Akt signaling, coordinated with HER3 and p85α in LK2/HER2 cells (see Fig. 6), and this signaling might be specifically affected by gefitinib. On the other hand, EGFR tyrosine kinase inhibitors often disrupt the formation of HER2/HER3 heterodimers in HER2-overexpressing cancer cells (25, 27), and we have shown that association of HER2 with HER3 in LK2/HER2 cells is markedly abrogated by gefitinib (Fig. 5). Expression of HER2 is essential for the dimer formation of HER2 and HER3, whereas exogenous addition of heregrin, a specific ligand for HER3, could not further enhance dimer formation or HER3 phosphorylation, in LK2/HER2 transfectants (data not shown). Treatment with gefitinib inhibited HER3 phosphorylation with release of p85α in LK2/HER2 cells under basal growth conditions. Concomitant disruption of HER2/HER3 formation by gefitinib, and the associated release of p85α, seems to specifically affect Akt signaling, resulting in growth arrest of cancer cells (Fig. 6).

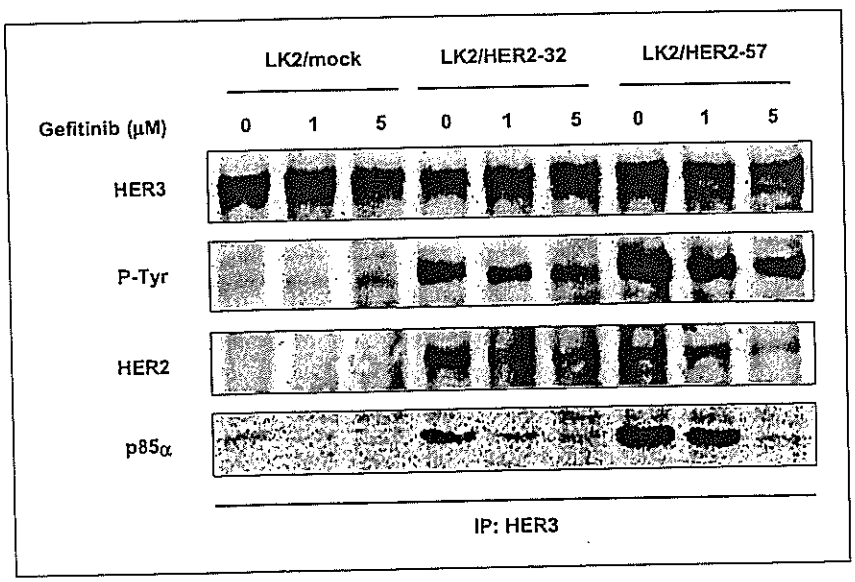


Figure 5. Gefitinib inhibits the formation of HER2/HER3 heterodimers and the association of HER3 with p85α in LK2/HER2 cells. Exponentially growing LK2/HER2 or LK2/mock in 10% serum medium were treated with gefitinib (0.1 or 5 μmol/L) for 3 hours. After cell lysis, HER3 was immunoprecipitated with specific antibody. The immunoprecipitates were divided equally and subjected to immunoblot analysis using the indicated antibodies against HER3, P-Tyr, HER2, and p85α, respectively.

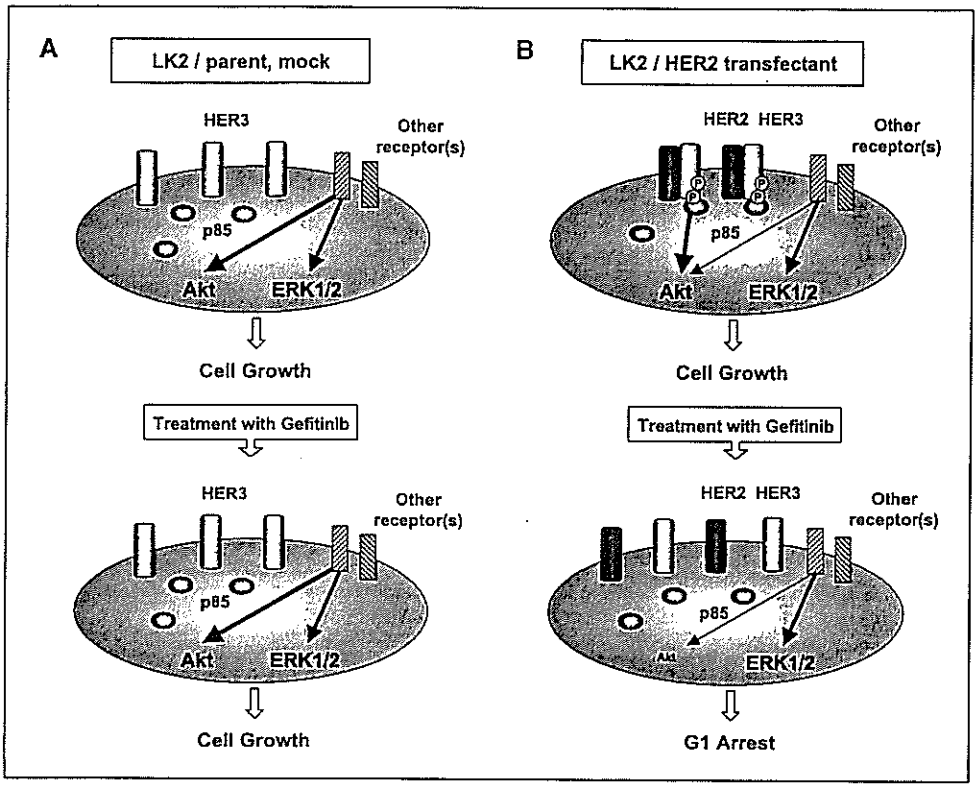


Figure 6. A hypothetical model of how drug sensitivity to gefitinib is controlled in HER2 expressing NSCLC cells. In gefitinib-resistant cell lines (LK2/mock and LK2/parent) with no significant expression of EGFR, EGFR is not a survival factor for these cells. Cell proliferation and apoptosis are expected to be driven by other growth factor receptors that might not be targets for gefitinib. On the other hand, in gefitinib-sensitive cell lines (LK2/HER2-32 and LK2/HER2-57), only HER2-driven signaling was dominant, following activation of Akt through the formation of heterodimers with HER3. Therefore, cell survival and death, both of which are dependent on HER2/HER3 signaling, are highly susceptible to gefitinib.

In conclusion a NSCLC cell line, LK2, has no apparent expression of EGFR and HER2, but expresses HER3 moderately, suggesting that HER2 (this study) as well as EGFR (23) does not seem to act as survival factors in LK2 cells. Cell proliferation and apoptosis in LK2 cells are expected to be driven by other growth factor receptors that are not targets for gefitinib (Fig. 6*A*). Overexpression of EGFR in LK2 cells resulted in no altered drug sensitivity to gefitinib (23). In contrast, overexpression of HER2 in LK2 cells results in enhanced drug sensitivity to gefitinib, and stimulates HER2-driven signaling accompanied by activation of Akt, plausibly through HER2/HER3 heterodimer formation (Fig. 6*B*). Cell survival and death, which are dependent on HER2/HER3 signaling, are then expected to be highly responsive to gefitinib treatment. Although our present study was done with artificially gene-modified cancer cells through wild-type HER2 cDNA transfection, Moasser et al. (24) have previously reported that gefitinib selectively inhibits HER2-driven signaling and suppress the growth of HER2-overexpressing breast and ovary cancer cell lines: these cancer cell lines were established from cancer patients without gene transfection. Further assessment of the activation levels of HER2 and/or HER3 could be useful in determining the therapeutic efficacy of the drug in a subgroup of NSCLC patients.

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Antimyeloma effects of a novel synthetic retinoid Am80 (Tamibarotene) through inhibition of angiogenesis

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In multiple myeloma (MM), the interaction between myeloma cells and bone marrow microenvironment has an important role in the pathogenesis of MM. We first examined the inducing effect of myeloma cells on migration of human umbilical vein vascular endothelial cells (HUVECs). Five myeloma cell lines produced varying amounts of VEGF, and migration of HUVECs was induced by coculture with myeloma cells. We next examined the inhibitory effect of a novel synthetic retinoid Am80 (Tamibarotene) on both myeloma cells and HUVECs. Am80 is specific for the retinoic-acid receptor-α/β, and has therapeutic effects in all-*trans* retinoic acid resistant acute promyelocytic leukemia. Am80 slightly inhibited the growth of both myeloma cells and HUVECs, and remarkably inhibited the growth of HUVECs stimulated by VEGF. Am80 showed little growth inhibition of bone marrow stromal cells (BMSCs), but it markedly inhibited migration of HUVECs by cocultured myeloma cells. Am80 inhibited VEGF-induced phosphorylation of VEGF receptor. In addition, VEGF-induced formation of tube-like structures *in vitro* and neovascularization in mouse corneas were significantly inhibited by Am80. These findings clearly demonstrate that Am80 is a potential inhibitor of angiogenesis caused by the interaction between vascular endothelial cells and myeloma cells, and might be a useful therapeutic agent against MM.

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Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by the clonal proliferation of terminally differentiated plasma cells, monoclonal gammaglobulinemia, immunodeficiency, renal insufficiency and multiple bone lesions. [1,2] Although high-dose chemotherapy induces complete remission in a small fraction of patients, long-term remission is rare. In addition, sufferers tend to belong to older age groups, which can limit the use of conventional chemotherapeutic agents due to complications. As a result, there is an urgent need for novel treatment modalities that are suitable even for high-risk patients.

The interaction between myeloma cells and bone marrow microenvironment has a key role in the pathogenesis of MM.[2,3] These involve the production of cytokines and growth factors such as interleukin-6 (IL-6) and insulin-like growth factor-1, as well as physical interactions. IL-6 strongly promotes the cellular proliferation of myeloma cells, especially in the early stages of tumorigenesis.[3,4] Myeloma cells produce several proangiogenic factors, including VEGF, fibroblast growth factor-2, angiopoietin-1, metalloproteinases and urokinase-type plasminogen activator.[5–7] Bone marrow angiogenesis has an important role in progression of MM: microvessel density and angiogenesis in the bone marrow are correlated with the prognosis and survival of MM patients.[5–7] Therefore, VEGF and its cognate receptor are both believed to be potential targets for antimyeloma treatment. Preclinical trials with VEGF-neutralizing antibodies have reported inhibition of the production of metalloproteinases, tumor necrosis factor-α and IL-1β in solid tumors[8] and myelomonocytic leukemia.[9] Moreover, VEGF receptor (VEGFR) tyrosine kinase inhibitor has been shown to suppress the proliferation and migration of myeloma cells expressing VEGFR-1 (Flt-1).[10] It is currently under a phase II study for the treatment of MM.[11] Thalidomide has been reported to inhibit the myeloma cell growth through induction of apoptosis and blockade of the interaction between myeloma cells and bone marrow stromal cells (BMSCs),[2] in addition to the antiangiogenic activity through inhibition on FGF-2 induced angiogenesis.[12] Thus, an antiangiogenic therapeutic strategy might be useful for the treatment of MM.

All-*trans* retinoic acid (ATRA) and other retinoids have been reported to inhibit the growth of a range of malignancies both *in vitro* and *in vivo*, including acute promyelocytic leukemia (APL), neuroblastoma, lung cancer and MM.[13–18] ATRA induces cell differentiation, cell cycle arrest, apoptotic cell death, and IL-6/IL-6R downregulation *in vitro*.[16–18] Retinoids exert their effects through interactions with two types of nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR), each of which has three subtypes (-α, -β and -γ). ATRA is one of the most clinically successful retinoids, however, its high rates of adverse effects, instability, and the appearance of ATRA-resistant leukemia cells have provided obstacles to ATRA-based therapies.[19] In an attempt to overcome these problems, Am80 has been synthesized by the introduction of heteroatoms into ATRA-like structures (Figure 1).[20] Am80 is a RAR-α/β-selective retinoid, that does not bind and activate RAR-γ and RXRs, hence can spare unfavorable adverse effects. Am80 has little affinity for cellular retinoic acid binding protein (CRABP) and is active against CRABP-rich ATRA-resistant cells.[21,22] In addition, it is more stable against light, heat and oxidation than ATRA. Tobita *et al* reported that 14 out of 24 (58%) APL patients who relapsed after ATRA treatment showed complete remission by the treatment with Am80 and experienced fewer adverse side effects in clinical trials.[22] Thus, Am80 has additional therapeutic advantages compared with ATRA. Furthermore, Am80 has been shown to decrease IL-1-induced IL-6 production *in vitro*,[23] and inhibit angiogenesis in chicken chorioallantoic membranes.[24] However, there have been no further reports on the mechanisms of this antiangiogenic activity.

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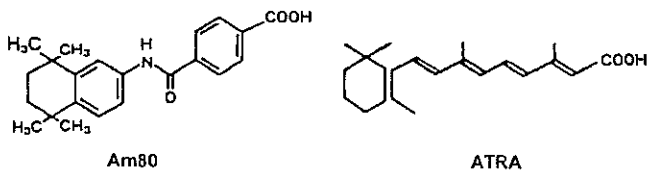

Figure 1 Chemical structure of Am80 and ATRA.

In this study, we therefore examined the inhibitory effects of Am80 on myeloma cell-induced angiogenesis.

Materials and methods

Cell lines and culture

The human myeloma cell lines, U266, AMO1, ILKM-2, ILKM-3 and ILKM-8, used in this study, were described previously.[25,26] All cell lines were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), streptomycin, and penicillin. Recombinant human IL-6 (Diaclone research, Besancon, France) was added at 10 ng/ml to the medium of ILKM-2, ILKM-3 and ILKM-8. Human umbilical vein vascular endothelial cells (HUVECs) were purchased from Clonetics (Walkers Ville, MD, USA) and BMSCs cells were from Bio Whittaker (MD, USA) through Sanko Junyaku (Tokyo, Japan), and were cultured according to the manufacturer's instruction. NIH3T3/KDR and NIH3T3/Flt-1 cells, which were kindly provided by Dr M Shibuya (University of Tokyo, Tokyo, Japan), were cultured in DMEM supplemented with 10% FBS.

Materials

Am80 was kindly gifted by Dr H Kagechika (University of Tokyo, Tokyo, Japan).[27] ATRA was purchased from Sigma (St Louis, MO, USA). Chemical structures of Am80 and ATRA are presented in Figure 1. Each retinoid was dissolved in dimethylsulfoxide at 10 mM. Human recombinant $VEGF_{165}$ was purchased from R&D Systems (Minneapolis, MN, USA). The selective VEGF receptor tyrosine kinase inhibitor, 3-[(2,4-dimetylpyrrol-5-yl) methylidenyl]-iodolin-2-one (SU5416) was provided by SUGEN[28] and the Taiho Pharmaceutical Co. (Tokyo, Japan).

Growth inhibition assay

The growth inhibitory effects of the retinoids for myeloma cell lines were determined using a methyl-thiazolyl tetrazolium (MTT) assay. Cells (2×10^4/well) were cultured in 96-well plates in triplicate with or without Am80, in a final volume of 100 μl at 37°C for 48 h. After incubation, 10 μl of 5 mg/ml MTT solution (Sigma) was added to each well, the cells were incubated for 4 h at 37°C, and 100 μl of lysis buffer (0.04 N HCl and isopropanol) was added. The optical densities (ODs) at 570 and 630 nm were measured with a multiplate reader. Cell growth (%) was calculated as the following: (OD_{630}–OD_{570} of the samples/ OD_{630}–OD_{570} of the control) × 100. For HUVECs or BMSCs, $2.5–3.0 \times 10^4$ cells were cultured in 24-well plates in triplicate under a basal growth condition or serum starved condition for 24 h and incubated with or without VEGF or Am80 for another 48 h. Then the cell number was counted by Coulter counter.

Enzyme-linked immunosorbent assay (ELISA) of VEGF for cocultured system

Myeloma cells (1×10^5) were cultured at 37°C in a final volume of 1 ml in culture medium. We used the conditioned medium for coculture migration assay after 48 h incubation and the concentration of VEGF was determined using commercially available ELISA kit (R&D systems).

Immunoblotting assay

NIH3T3/KDR and NIH3T3/Flt-1 cells were cultured in serum-depleted DMEM for 48 h and then cells were preincubated with Am80 for 12 h or SU5416[28–30] for 3 h followed by stimulation with 30 ng/ml VEGF for 5 min at 37°C. After incubation, the cells were washed with cold-PBS (phosphate-buffered saline) and lysed in the buffer (50 mM HEPES, 150 mM NaCl, 1% Triton X-100, and 10% glycerol containing 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM sodium vanadate). Cell lysates were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore, MA, USA). After transfer, membranes were incubated with the blocking solution and probed with antiphosphotyrosine (PY20), anti-KDR or anti-Flt-1 antibody (Santa Cruz Biotechnologies, CA, USA), antiphospho ERK1/2 or anti-ERK1/2, antiphospho Akt or anti-Akt antibody (Cell Signaling, MA, USA) followed by washing. The protein contents were visualized using horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (ECL, Amersham/Pharmacia Biotech, NJ, USA).

Migration assay under coculture system

To assay for migration of HUVECs or BMSCs under coculture system, HUVECs or stromal cells were plated in the inner chambers after preincubation with or without Am80 or ATRA for 12 h or SU5416 for 3 h, and followed cocultivation with myeloma cells in the outer chambers (see Figure 2b). HUVECs (3×10^5) or stromal cells (3×10^5) were suspended in medium and seeded in the inner chamber with polycarbonate filters (8 μm pores: Kurabo Inc., Tokyo, Japan) coated with human plasma fibronectin (1.33 μg/ml: Life Technologies, Inc., Tokyo, Japan). After 6 h (HUVECs) or 12 h (stromal cells) incubation at 37°C, media in the inner chambers were aspirated, and cells on the upper surface of the filters were removed with a cotton swab. Cells on the lower surface of the filters were fixed with ethanol and stained with Giemsa, and the stained nuclei were counted. Five fields (× 100) per chamber were counted, and average numbers were determined with three chambers.

In vitro *tube-formation assay*

The effect of Am80 and ATRA on *in vitro* tube formation was assessed based on the VEGF-induced tube formation of HUVECs using an angiogenesis kit (Kurabo Industries Ltd, Tokyo, Japan) according to the manufacture's instructions. Briefly, cocultured HUVECs and fibroblasts were incubated with or without 10 ng/ml VEGF and serial dilutions of Am80 or ATRA for 11 days. The medium containing VEGF and Am80 or ATRA was changed every 3 days. The wells were then fixed and immunostained with CD31, and the stained capillary structures in each well were photographed and quantified.

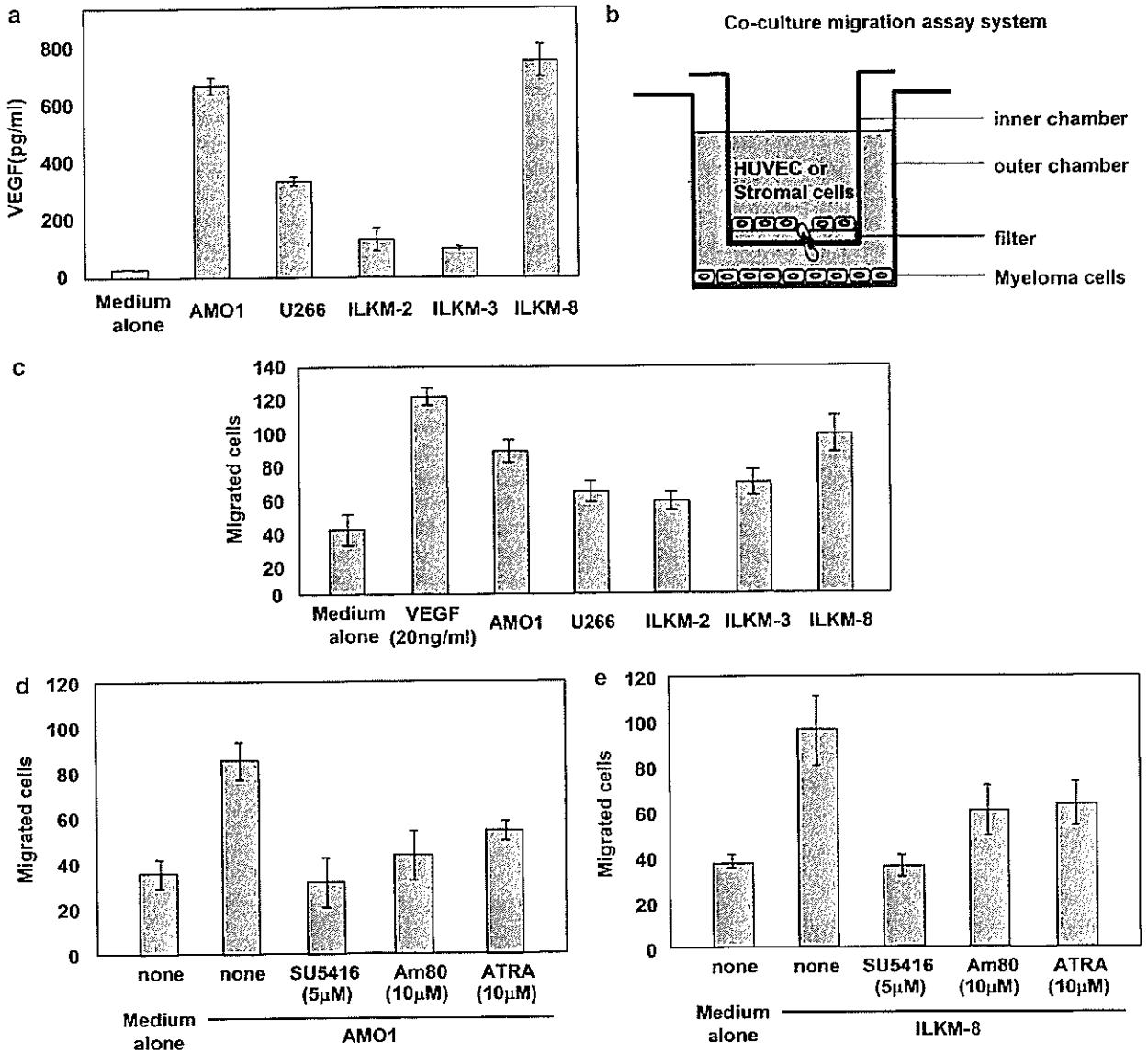

Figure 2 Interaction between vascular endothelial cells and myeloma cells. (a) Production of VEGF by five myeloma cell lines (AMO1, U266, ILKM-2, ILKM-3 and ILKM-8). Myeloma cells (1×10^5) were incubated for 48 h in the presence of IL-6, and VEGF contents in culture medium were determined by ELISA. Each value is the mean ± standard deviation (s.d.) of triplicate cultures. (b) Schemes of migration assay under coculture system. Myeloma cells were preincubated in the outer chambers and then HUVECs or BMSCs were suspended in medium and seeded in the inner chamber. (c) Migration of HUVECs cocultured with myeloma cells. HUVECs were cocultured with myeloma cells for 6 h and migration was shown as the mean ± s.d. of triplicate cultures. (d, e) Inhibitory effects of Am80, SU5416 and ATRA on migration of HUVECs cocultured with AMO1 (d) and ILKM-8 (e). HUVECs were pre-treated with or without Am80 or ATRA for 12 h, or with SU5416 for 3 h, and then cocultured with myeloma cells for 6 h. After incubation, migrated cells were counted after Giemsa staining.

Mouse corneal micropocket assay and quantification of corneal neovascularization

A mouse corneal micropocket assay was performed as described previously.[29,30] Briefly, 0.3 μl of hydron pellets (IFN Sciences, NJ, USA) containing VEGF (200 ng/pellet) was implanted into the corneas of male BALB/c mice on day 1. Am80 was administered orally and SU5416 was administered intraperitoneally on days 0–6. All mice were killed on day 6 and their corneal vessels were photographed. Images of the corneas were recorded using Nikon Coolscan software (Nikon Corp., Tokyo, Japan) and corneal neovascularization was analyzed using the NIH Image 1.61 software package.[31]

Results

Migration of vascular endothelial cells cocultured with myeloma cells and inhibitory effect of Am80

We first examined the inducing effect of myeloma cells on migration of HUVECs. As shown in Figure 2a, five myeloma cell lines secreted varying amounts of VEGF into the culture medium during incubation for 48 h. We then examined if myeloma cells could stimulate migration of HUVECs under coculture assay condition (Figure 2b). The migration of HUVECs to the lower surface of the filters was assayed after incubation for further 6 h. The results revealed that coculture with AMO1 or ILKM-8,

which secreted large amounts of VEGF, induced about a two to three-fold increase in migration compared with the control in the absence of myeloma cells (Figure 2c). When cocultured with U266, ILKM-2 or ILKM-3, which produced less amount of VEGF than AMO1 or ILKM-8, migration of HUVECs showed much less increase. Remarkable cell migration of HUVECs was observed by exogenous addition of VEGF (20 ng/ml) in the outer chamber.

We then examined the inhibitory effect of Am80, ATRA and SU5416 on migration of HUVECs when cocultured with AMO1 or ILKM-8 cells. Chemical structures of Am80 and ATRA are shown in Figure 1. Myeloma cell-induced migration of HUVECs was remarkably inhibited by Am80 at 10 μM (Figure 2d and e). ATRA also showed the inhibitory effect at the similar levels to Am80, and SU5416, a selective VEGF receptor tyrosine kinase inhibitor, inhibited migration of HUVECs at 5 μM completely.

Growth inhibitory effect of Am80 on myeloma cells and HUVECs

We then examined if Am80 inhibited the growth of myeloma cells and HUVECs. Am80 at 10 μM showed a subtle inhibitory effect on the basal growth of myeloma cells and HUVECs (Figure 3a and b). Mean of 50% inhibitory concentration (IC_{50}) values of Am80 were ranging from 20 to 30 μM. We also examined the growth inhibitory effect of Am80 on HUVECs under VEGF-stimulated condition (Figure 3c). Exogenous addition of VEGF stimulated cell growth more than 1.5-fold over the control, and Am80 significantly inhibited VEGF-induced cellular growth of HUVECs (IC_{50} values are 5 μM).

Am80 inhibits migration of BMSCs cocultured with myeloma cells

In order to understand if Am80 could modulate the microenvironment in multiple myeloma, we then examined the effect of Am80 on cell growth and migration of BMSCs. As shown in Figure 4a, Am80 up to 10 μM was found to show no inhibition on cell growth of BMSCs. Coculture with ILKM-8 or AMO1 stimulated migration of stromal cells two- to three-fold over the control, and Am80 inhibited myeloma cell-induced migration in a dose-dependent manner (Figure 4b).

Am80 induces IL-6/IL-6R downregulation and cell cycle arrest in myeloma cell

We then examined the mechanism of the growth inhibitory effects of Am80 on myeloma cells (see Supplementary Information). The IL-6 production from U266 cells was decreased by the treatment of Am80 in a dose-dependent manner, and IL-6R expression on cell surface membrane of U266 and ILKM-2 cells was also downregulated by Am80 at 1 μM after 24 h. Regarding cell cycle progression, the number of cells at the G0/G1 phase was slightly increased, and that at the S and G2/M phases was reduced in both ILKM-2 and U266 cells after 24 h treatment with Am80 at 1 μM. We further examined the expression and phosphorylation of cell cycle-related proteins using immunoblotting. The levels of phosphorylated Rb markedly decreased 24 h after treatment with Am80 at 1 μM, and a slight increase in p21 levels was observed. Apoptotic changes were not observed (data not shown).

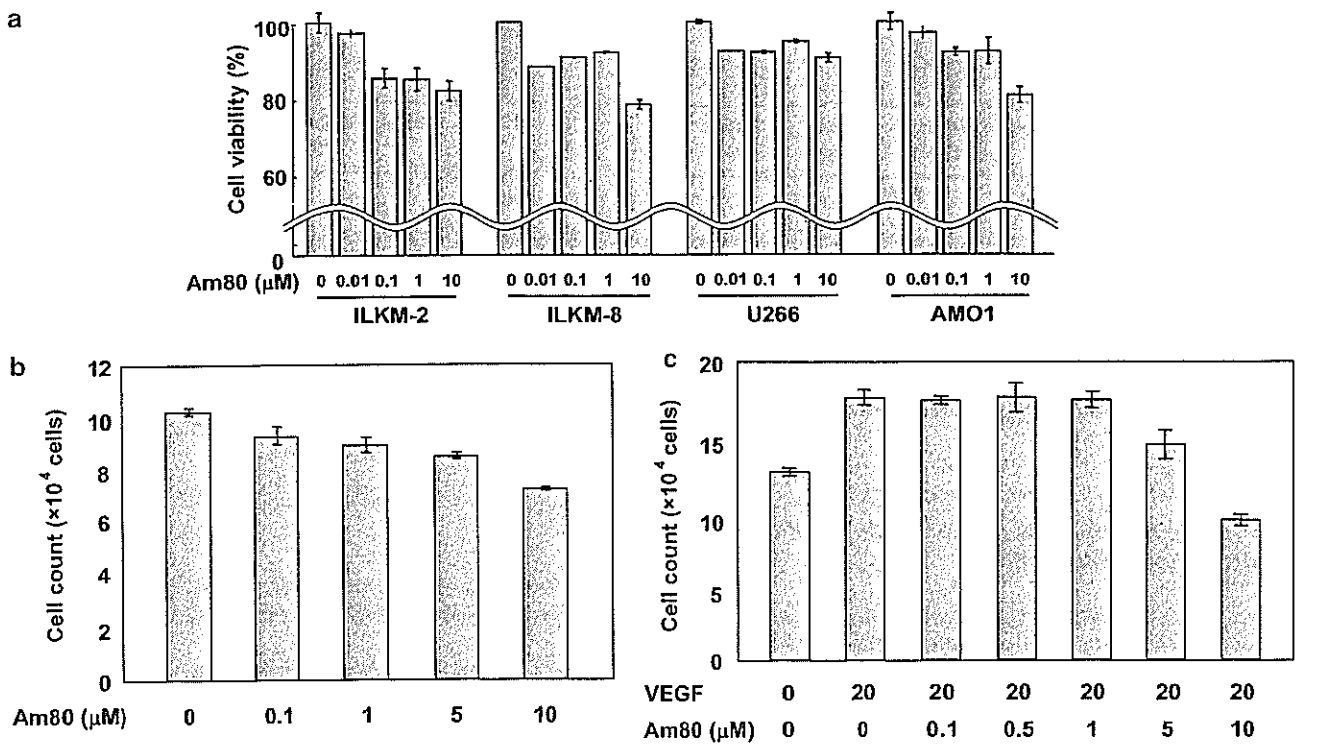

Figure 3 Inhibitory effect of Am80 on growth of myeloma cell lines and HUVECs. Five myeloma cell lines or HUVECs were incubated with or without various concentration of Am80 for 48 h. The results are shown as the mean±s.d. of triplicate cultures. (a) Direct growth inhibition of myeloma cells by Am80. Myeloma cells were incubated with Am80 and cell growth was measured using an MTT assay. Cell growth (%) is indicated as a percentage compared with the untreated control. (b, c) Growth inhibition of HUVECs by Am80 under basal condition (b) or under VEGF-stimulated condition (c). Cell number was counted by Coulter counter.

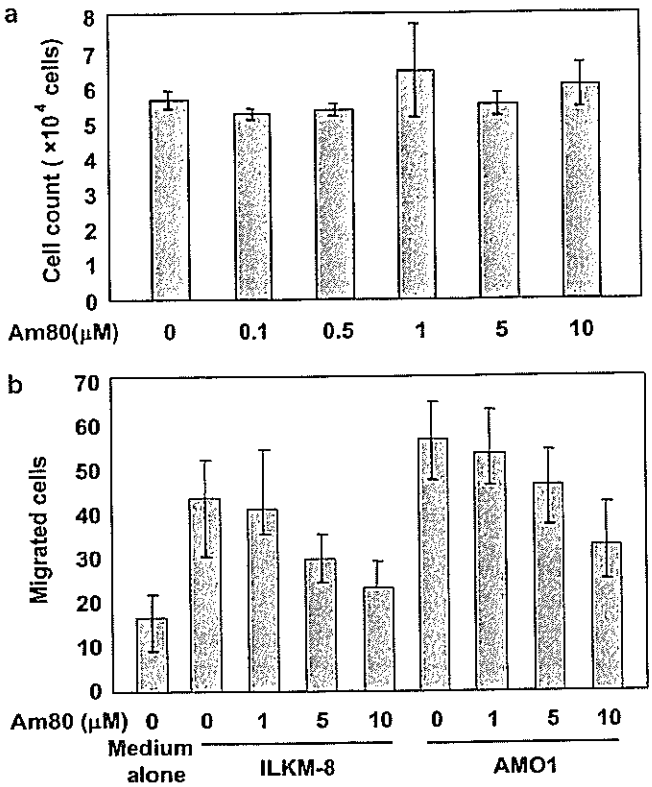

Figure 4 Inhibitory effect of Am80 on growth or migration of BMSCs. (a) Cell growth inhibition of BMSCs by Am80. BMSCs were incubated with or without Am80 for 24 h and cell number was counted by Coulter counter. (b) Inhibitory effect of Am80 on cell migration of BMSCs cocultured with ILKM-8 or AMO1. Myeloma cells (1×10^5 cells) were cocultured with pretreated stromal cells with Am80 and incubated for 12 h. Migrated cells were counted after Giemsa staining as the mean ± s.d. of triplicate cultures.

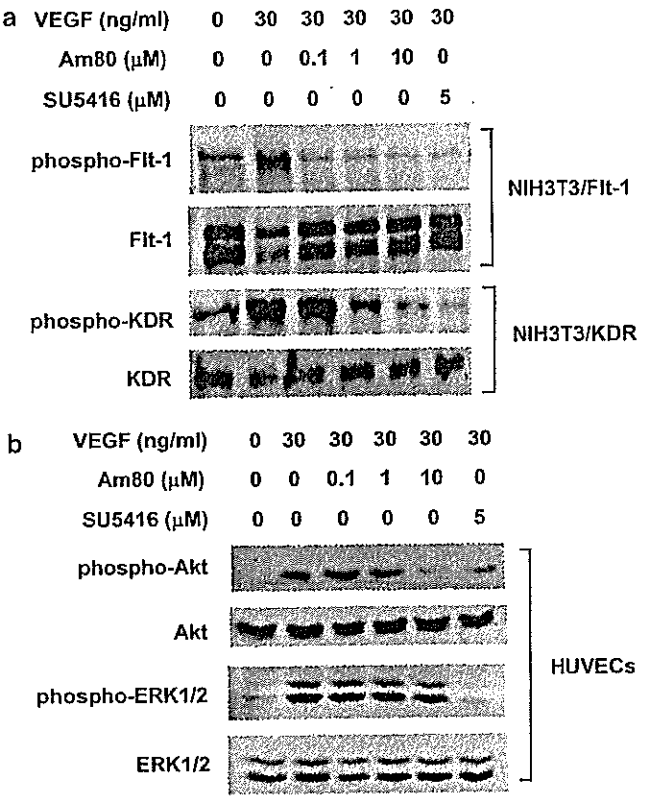

Figure 5 Effect of Am80 or SU5416 on VEGF-induced phosphorylation of KDR and Flt-1, and ERK1/2 and Akt. (a) NIH3T3/KDR, NIH3T3/Flt-1 cells were grown to near confluence and then serum depleted for 48 h. The cells were preincubated with indicated concentrations of Am80 for 12 h or SU5416 for 3 h, followed by the addition of 30 ng/ml VEGF for 5 min at 37°C. Protein extracts were resolved by SDS-PAGE, and Flt-1 and KDR were probed with antiphosphotyrosine, anti-Flt-1, or anti-KDR antibody. (b) Protein extracts of HUVECs were probed with antiphospho Akt, anti-Akt, antiphospho ERK1/2 or anti-ERK1/2 antibody (b).

Effect of Am80 on VEGF-induced autophosphorylation of VEGF receptors

In order to understand the inhibitory mechanism of Am80 on VEGF-induced migration of HUVECs, we examined if Am80 could modulate VEGF-induced activation of two VEGF receptor molecules, VEGFR-1(Flt-1) and VEGFR-2(KDR). We could not detect any apparent expressions of both VEGFRs in HUVECs by immunoblotting, since the receptor numbers were very low. We therefore used Flt-1 or KDR overexpressing NIH3T3 cells. In these cells, immunoblotting with antiphosphotyrosine antibody indicated that Flt-1 and KDR were autophosphorylated in response to VEGF (Figure 5a), consistent with our previous report.[30] Am80 significantly inhibited autophophorylation of Flt-1 at 0.1 μM, while Am80 inhibited that of KDR at 1 μM (Figure 5a). SU5416 completely blocked autophosphorylation of both VEGFRs at 5 μM. We also examined the effect of Am80 on the downstream signaling molecules of VEGF receptors, Akt and ERK1/2 in HUVECs. We could detect apparent activation of Akt and ERK1/2 in response to VEGF in HUVECs (Figure 5b). Phosphorylation of Akt was almost completely inhibited by Am80 at 10 μM. However phosphorylation of ERK1/2, however, was not inhibited by Am80 at 10 μM. By contrast, SU5416 at 5 μM almost completely inhibited phosphorylation of both Akt and ERK1/2. We also examined the effect of Am80 on VEGF production by myeloma cells, however, Am80 did not affect VEGF production (data not shown).

Effect of Am80 on VEGF-induced tube formation by vascular endothelial cells

We then examined if Am80 inhibited the formation of tube-like structures *in vitro* in response to VEGF using HUVECs. As shown in Figure 6A, 10 ng/ml of VEGF increased tube formation, whereas it was significantly inhibited by Am80 at 10 μM. Am80 did not show any cytotoxic effects even at a dose of 10 μM in this condition using confluent HUVECs. Quantitative analysis demonstrated that the rate of tube formation was two-fold higher in the presence of VEGF, but was inhibited by Am80 in a dose-dependent manner (Figure 6B). Am80 at 0.1 or 1 μM showed almost 40–50 or 50–60% inhibition, respectively. ATRA also inhibited tube formation (Figure 6C), but cytotoxic effect was detected at a dose of 10 μM (data not shown).

Am80 inhibits VEGF-induced neovascularization in the mouse cornea

We finally examined the *in vivo* effect of Am80 on VEGF-induced angiogenesis using a mouse corneal micropocket assay.

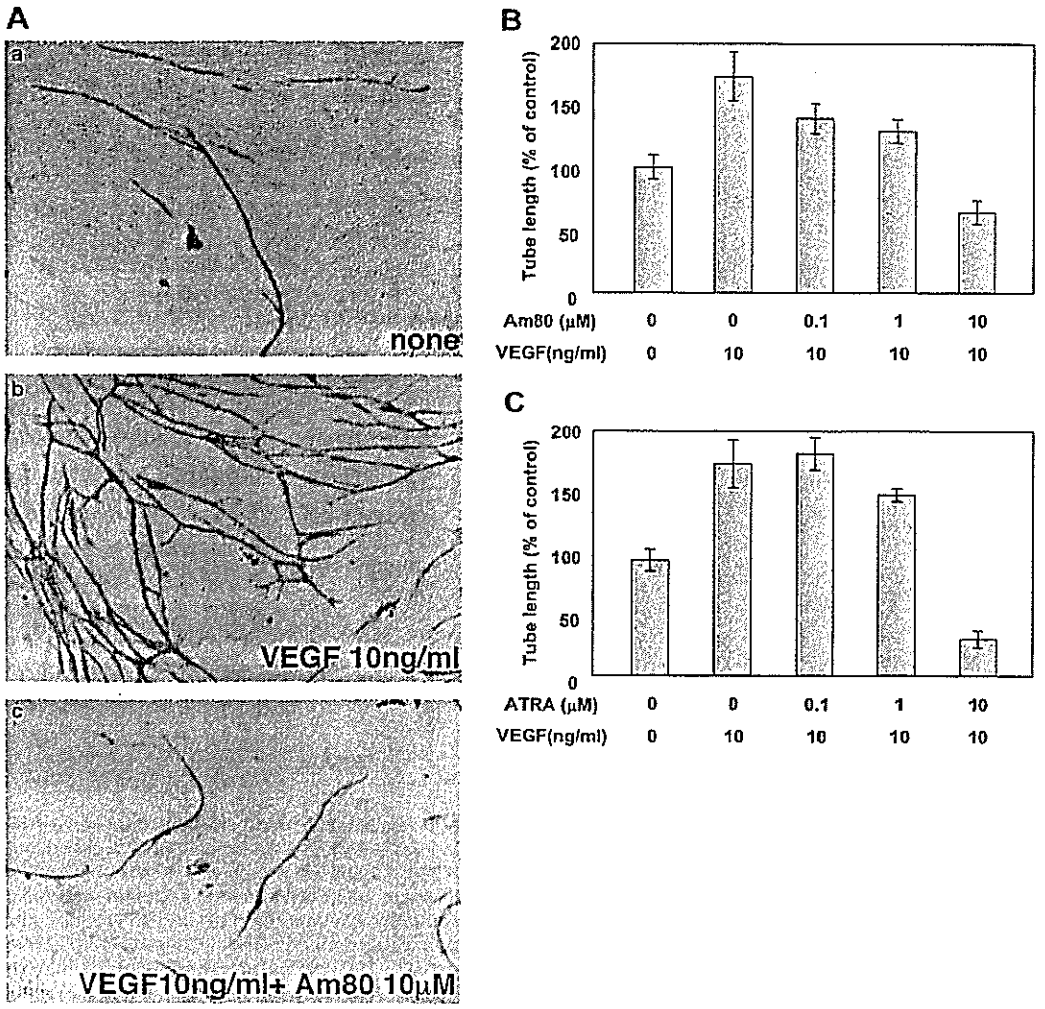

Figure 6 VEGF-induced formation of tube-like structures and inhibitory effect of Am80. (A) The development of tube-like structures after 11 day's incubation in the absence or presence of VEGF (10 ng/ml); tube formation was significantly inhibited by Am80 at 10 μM without inhibition of cell viability. Quantitative analysis of the dose-dependent effect of Am80 (B) and ATRA (C). Tube formation was quantified as described in the Materials and methods. The results were shown as the mean $\pm$ s.d. of triplicate analyses.

VEGF (200 ng) in a hydron pellet-induced angiogenesis in the mouse cornea (Figure 7A and B). Oral administration of Am80 (10 mg/kg/day) significantly inhibited the VEGF-induced angiogenesis (Figure 7A a–c). Intrapenitonial administration of SU5416 (25 mg/kg/day) also inhibited VEGF-induced neovascularization consistent with our previous findings (Figure 7A d and B).[29–31]

Discussion

In spite of a significant advancement in conventional chemotherapy and wider applicability of high-dose treatment with transplantation of hematopoietic stem cells, MM still remains incurable. [1,2] It is recently reported that the interaction between myeloma cells and bone marrow microenvironment have crucial roles in the pathogenesis of MM,[2] and acquired drug resistance is also mediated by environmental factors.[32] Multiple myeloma cells could stimulate angiogenesis through paracrine control of VEGF and other proangiogenic factors. Therefore, we examined the myeloma cell-dependent angiogenesis and inhibitory effect of Am80 *in vitro* and *in vivo*.

Myeloma cell lines produced various amounts of VEGF, and cell migration of HUVECs was promoted by VEGF or coculture with myeloma cells (Figure 2). Am80 showed subtle (for myeloma cells and HUVECs) or little (for BMSCs) inhibitory effects on proliferation under basal condition (Figures 3 and 4a). However, VEGF-induced proliferation of HUVECs was significantly inhibited by Am80 at 10 μM (Figure 3c). Moreover, Am80 markedly inhibited migration of both HUVECs (Figure 2d and e) and BMSCs (Figure 4b) when cocultured with myeloma cells. Am80 also blocked VEGF-induced formation of tube-like structures *in vitro* (Figure 6) as well as neovascularization in mice corneas without cytotoxic effect (Figure 7). In addition, Am80 inhibited the phosphorylation of both VEGFR-1 and VEGFR-2 at similar levels as SU5416 (Figure 5). Am80 did not affect VEGF production by myeloma cells under the coculture condition (data not shown). Although further investigation about the molecular mechanisms is necessary, these results demonstrated that Am80 has an inhibitory effect on VEGF- or myeloma cell-induced angiogenesis both *in vitro* and *in vivo* through downregulation of VEGF/VEGFR signaling. Therefore, Am80 is a potent inhibitor of this signaling, which might be a useful therapeutic agent against MM (Figure 8).

Concerning the clinical use of retinoid for MM, several trials have been evaluated. Vesole *et al* (University of Arkansas) followed 11 MM patients receiving ATRA therapy, all of them were heavily pretreated and resistant to conventional chemotherapy (Vasole *et al*, personal communication). None of the patients achieved a complete or partial response (0%), one patient experienced stable disease (9%) and 10 patients were classified with progressive disease (91%). Koskela *et al*[33] also reported that ATRA alone is not effective to pretreated MM patients. These results were not satisfactory, however, all of the enrolled patients had heavily treated refractory myeloma, suggesting that tumor cells had lost their dependence of growth on IL-6. In this study, we observed the subtle inhibitory effect on myeloma cell growth through cell cycle arrest, as well as downregulation of IL-6/IL-6R (Figure 3a and Supplementary Information). IL-6/IL-6R signaling is much more important for the proliferation of early stage MM than refractory one.[1] Therefore, although both Am80 and ATRA do not have sufficient antitumor effect against cell lines or refractory MM, it is not possible to conclude that all types of retinoids will be ineffective against newly diagnosed or early stage MM. Furthermore, the pharmacological effects of retinoids are strongly affected by their chemical stability and by drug resistance in cancer cells. Serum level of Am80 was found to be more stable in patients than ATRA (unpublished data), and Am80 is characterized by a low potential for drug resistance, minimal adverse events and superior chemical stability. In high-risk patients, it is important that the risk of therapy-related mortality does not exceed the potential advantages of a treatment. Since Am80 appears to be the safe and practical agent, it could be used as the chemopreventive agents in combination with other chemotherapeutic or noncytotoxic agents for the indolent and smoldering types of myeloma cells, which do not necessarily need intensive chemotherapy and maintains the sensitivity to IL-6. In addition, it would be a suitable agent especially for the initially diagnosed MM in elderly, who cannot receive cytotoxic therapies. We have examined the synergistic inhibitory effect of Am80 on myeloma cell in combination with dexamethasone, and confirmed that Am80 possessed an additive effect on dexamethasone (data not shown).

Our results clearly show that Am80 has an inhibitory effect on angiogenesis induced by the interaction between vascular

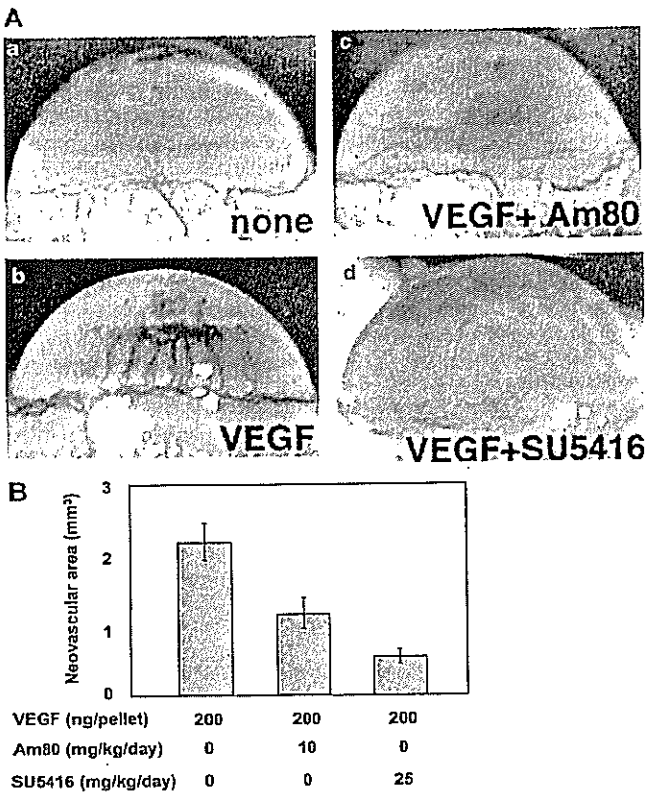

Figure 7 VEGF-induced angiogenesis in mouse corneas and inhibitory effect of Am80. (A) Photographs of angiogenesis in mouse corneas in the absence or presence of hydron pellets containing VEGF (200 ng/pellet), after oral administration of Am80 (10 mg/kg/day) or intraperitoneal administration of SU5416 (25 mg/kg/day) for 6 days. (B) Quantitative analysis of mouse corneal angiogenesis. Neovascular areas were quantified as described in the Materials and methods. Columns show the mean ± s.d. of three or four independent experiments.

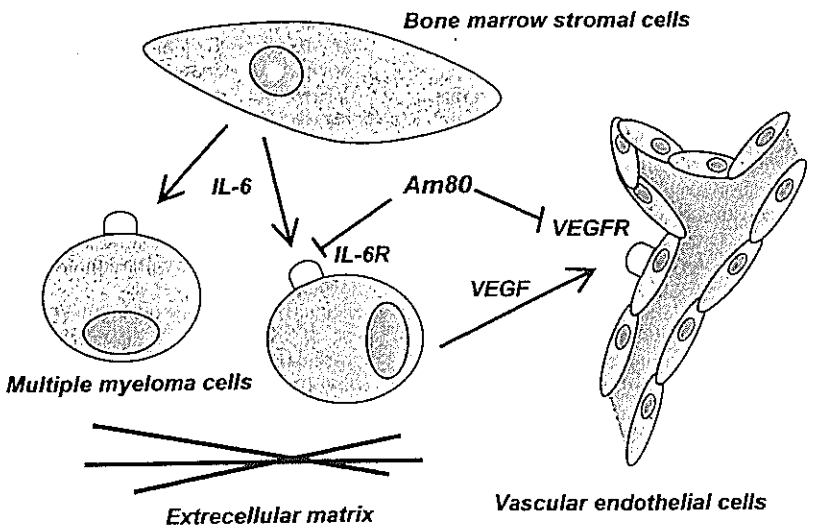

Figure 8 The scheme for inhibitory effects of Am80 on myeloma cell-induced angiogenesis. Multiple myeloma cells promote migration of vascular endothelial cells and angiogenesis through paracrine control of VEGF. Bone marrow stromal cells produce IL-6, which strongly stimulates cellular growth of myeloma cells. Am80 blocks myeloma cell-induced angiogenesis through inhibition of VEGFR phosphorylation. In addition, Am80 inhibits the expression of IL-6R on cell surface membrane of myeloma cells.