Proliferation of Human Neuroblastomas Mediated by the Epidermal Growth Factor Receptor

Ruth Ho, Jane E. Minturn, Tomoro Hishiki, Huaqing Zhao, Qun Wang, Avital Cnaan, John Maris, Audrey E. Evans, and Garrett M. Brodeur

Division of Oncology, Children's Hospital of Philadelphia and University of Pennsylvania, Philadelphia, Pennsylvania

Abstract

Neuroblastoma is a common solid tumor of childhood that is derived from the neural crest. Expression of epidermal growth factor (EGF) receptors (EGFRs) has been associated with enhanced cell growth and aggressive behavior in other tumors. Here, we examined the expression profile of EGFRs in neuroblastoma cell lines and primary tumors. We found that all 13 neuroblastoma cell lines examined expressed *EGFR1* (*HER1*), most at readily detectable levels. Low levels of other human EGFR family receptors were also detected in almost all cell lines. All primary tumors examined expressed readily detectable levels of *HER1* and *HER3* and lower levels of *HER2* and *HER4*. EGF had a significant effect on the proliferation of neuroblastoma cell lines *in vitro*. EGF treatment (100 ng/mL) of the cell lines SY5Y and NLF significantly increased cell number ($P < 0.01$). EGF stimulated more cells to enter S and G_2-M phase, as suggested by flow cytometry, indicating that EGF increases cell number by increasing proliferation, with no appreciable change in apoptosis. EGF exposure resulted in receptor autophosphorylation and activation of both the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways. Exposure to 0.5 μmol/L ZD1839, a HER1-specific inhibitor, caused a 40% to 50% reduction in the number of SY5Y and NLF cells grown in medium containing 10% fetal bovine serum ($P < 0.01$). Even at 0.01 μmol/L, ZD1839 inhibited autophosphorylation of HER1 by EGF. At 0.1 μmol/L, it also blocked phosphorylation of AKT, but not MAPK, in NLF cells. Additional studies showed that the PI3K/AKT–specific inhibitor LY294002 had a more profound effect than the MAPK-specific inhibitor U0126 in blocking EGF-induced cell proliferation. This suggests that the PI3K/AKT pathway is the main signaling pathway responsible for the proliferation effects of EGF in neuroblastomas. Our results also indicate that ZD1839 is a potent inhibitor of neuroblastoma cell proliferation; therefore, it may be a useful, biologically based therapeutic agent for these tumors. (Cancer Res 2005; 65(21): 9868-75)

Introduction

Neuroblastoma is the most common extracranial solid tumor in children. It arises from neural crest cells, and the tumors are most commonly found in the adrenal medulla or along the sympathetic chain (1). Neuroblastomas are heterogeneous both clinically and biologically. Tumors in infants may regress spontaneously, whereas tumors in older patients may mature into benign ganglioneuromas. Unfortunately, the majority of tumors occur between the ages of 1 and 5 years, and these tumors are generally unresectable or metastatic at the time of diagnosis and have a poor prognosis. Although intensive multimodality therapy has produced some improvements in the overall cure rate of these patients, this therapy has considerable short- and long-term toxicities. Therefore, a better understanding of the molecular pathogenesis of neuroblastomas may lead to biologically based therapy that is more effective and less toxic.

Tyrosine kinase receptors play an important role in survival, growth, and differentiation of many normal and malignant cells. For example, TrkA, the receptor for nerve growth factor, is commonly expressed in the most favorable neuroblastomas (2–4). Conversely, TrkB, along with its ligand, brain-derived neurotrophic factor (BDNF), is expressed in the more aggressive tumors, particularly those with *MYCN* amplification (5). TrkC, the receptor for neurotrophin-3, is expressed in a subset of TrkA-expressing tumors and is similarly associated with favorable clinical features and outcome (6, 7). Evidence suggests that the Trk family of tyrosine kinase receptors plays an important role in the behavior of both favorable and unfavorable neuroblastomas. However, the ultimate behavior of these tumors is probably dependent on additional pathways that also affect growth, survival, or differentiation.

The human epidermal growth factor (EGF) receptor (EGFR/HER1, also ErbB1), a 170 kDa transmembrane tyrosine kinase receptor, is one of four members of the human EGFR (HER) family receptors. The three other members are HER2 (Neu/ErbB2), HER3 (ErbB3), and HER4 (ErbB4). HER1 becomes biologically functional by forming a homodimer with itself or a heterodimer with other members of the HER receptor family. The mechanisms that promote the formation of receptor dimers include ligand binding and high receptor density due to overexpression. Ligands that can activate HER1 include EGF, amphiregulin, and transforming growth factor-α, which bind specifically to HER1, as well as β-cellulin, heparin-binding EGF, and epiregulin, which bind both HER1 and HER4 (8, 9).

HER1 is normally expressed in all epithelial and stromal cells but more selectively expressed in glial and smooth muscle cells. High levels of EGFRs have been observed in a variety of tumors, including head and neck, non–small cell lung, prostate, breast, gastric colorectal, and ovarian cancers. The expression of EGFRs in tumors has been correlated with tumor progression, poor survival, poor response to therapy, and resistance to cytotoxic agents. Constitutive activation of the EGFR pathway by gene amplification or other mechanisms may contribute to enhanced survival and growth of tumor cells (8, 10, 11).

HER family receptors are also important in the developing nervous system and in nervous system tumors. The HER2 and

Requests for reprints: Garrett M. Brodeur, Division of Oncology, Children's Hospital of Philadelphia, Abramson Research Center, Room 902-D, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318. Phone: 215-590-2817; Fax: 215-590-3770; E-mail: brodeur@email.chop.edu.

doi:10.1158/0008-5472.CAN-04-2426

HER3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system (12). HER3 and HER4 are coexpressed in childhood medulloblastoma, and their expression is associated with high proliferative activity in ependymoma (13, 14). Overexpression of HER1 is also found in glioblastoma multiforme (15).

There are a few reports concerning the expression of functional HER1 in neuroblastoma cell lines (16–18). However, the expression profile of HER family members in neuroblastoma cell lines and primary tumors, as well as downstream signaling pathways, have not been explored in detail. Therefore, we examined the expression and function of the EGFR pathway in neuroblastomas and determined whether EGFR-specific inhibitors might have a role as novel, biologically based therapeutic agents for neuroblastoma.

Materials and Methods

Cell lines and tumor samples. All cell lines (*MYCN* nonamplified, $n = 4$; *MYCN* amplified, $n = 9$) were obtained from the Children's Hospital of Philadelphia cell line bank (19). The lines were maintained at 5% CO_2 in RPMI 1640 with 10% fetal bovine serum (FBS; same lot used for all studies), 1% oxaloacete-pyruvate-insulin supplement, 1% glutamate, and 50 μg/mL gentamicin. A431, a human epidermoid carcinoma cell line that amplifies and overexpresses HER1 (20), was used as a positive control and grown under similar conditions. Cells were harvested when they reached 70-80% confluence to make RNA or protein. Eighteen neuroblastoma tumor samples were obtained from the Children's Hospital of Philadelphia tumor bank. Ten patients had biological favorable disease (ganglioneuroma, $n = 1$; stage I, $n = 3$; stage II, $n = 4$, stage IV-S, $n = 2$) and eight patients had biological unfavorable disease (stage III, $n = 4$; stage IV, $n = 4$).

Semiquantitative reverse transcription-PCR of *HER* family genes. Total RNA was extracted from neuroblastoma primary tumors and cell lines. Human fetal brain RNA was purchased from Stratagene (La Jolla, CA). Semiquantitative reverse transcription-PCR (RT-PCR) was carried out according to a previously described protocol (21). Primers for human *HER1* are 5′-CAGCGCTACCTTGTCATTCA-3′ and 3′-AGGACTCGAGAGACTCACGT-5′ (PCR product size: 195 bp); for human *HER2*, 5′-CTGACCAGTGTGTGGCCTGT-3′ and 3′-GACTGTGGATCGCCTCGCTA-5′ (PCR product size: 376 bp); for human *HER3*, 5′-ATGTGTGCCTTTGTTTCCCATC-3′ and 3′-GTGTTTCCCTTCAGCCCGTA-5′ (PCR product size: 293 bp); and for human *HER4*, 5′-GATGGAGGTTTTGCTGAAC-3′ and 3′-GGATACGCTCTGTTTGGGTTTG-5′ (PCR product size: 277 bp). The primers were biotinylated at their 5′ ends. PCR reactions were carried for 22 to 24 cycles. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was selected as the internal control because it has been the most consistent compared with others, such as *β-2-microglobulin, midkine, actin*, and *18S*, according to the semiquantitative and quantitative RT-PCR (Taqman) and microarray analysis data from our laboratory.

Cell viability assay. To study the effect of EGF on neuroblastoma, we used one cell line with moderate HER1 expression (SY5Y) and one with high HER1 expression (NLF). Cells were seeded into 24-well plates at a density of 5×10^3 per well in RPMI with 2% FBS overnight. Cells were treated the following day with 0 or 100 ng/mL EGF in 2% FBS-RPMI. To quantitate the number of viable cells, a colorimetric [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] assay was done at days 0 (after seeding), 2, 5, and 7 as previously described (1). A multiple wavelength scanner was used to measure the absorbance at 570 to 630 nm dual wavelengths. The experiments were done in five replicates and repeated four to five times. Measurement of absorbance was used as a correlate of viable cell number.

Flow cytometry. We used flow cytometric analysis of DNA content to decide whether EGF enhances growth by increasing proliferation or decreasing apoptosis. Cells were seeded in medium containing 2% FBS and treated with EGF (100 μg/mL) overnight, with or without pretreatment of ZD 1839 for 30 minutes. Cells were collected using 0.5% trypsin and resuspended in cold PBS at a concentration of 1×10^6 per milliliter. One-tenth volume of 10× propidium iodide solution (500 μg/mL propidium iodide, 10 mg/mL sodium citrate, and 1% v/v Triton X-100) was added, and cells were acquired in a flow cytometer within half an hour. A cell fit program (CELLQuest version 5.1, Becton Dickinson Bioscience, San Jose, CA) was used to calculate the fraction of cells with 2n, 2n-4n, and 4n amounts of DNA, corresponding to G_0-G_1, S, and G_2 + M phases of the cell cycle. Fractions of cells in different cell cycle phases under various conditions were compared.

Immunoblotting. To analyze the inhibition of HER1 autophosphorylation by ZD1839 (Iressa, AstraZeneca, Cheshire, United Kingdom), cells were grown in 10 cm^2 dishes to 80% confluence in standard culture medium. Cells were then grown in serum-free medium for a minimum of 4 hours before treatment with various concentrations (0.01, 0.1, 1, and 10 μmol/L) of the EGFR inhibitor ZD1839 for 1 hour. Cells were subsequently treated with EGF (10 or 100 ng/mL) for 5 minutes. To analyze inhibition of the PI3K/AKT and mitogen-activated protein kinase (MAPK) pathways, cells were pretreated with LY294002 or U0126 for 30 minutes, followed by EGF exposure (100 ng/mL) for 5 minutes. The cell pellets were lysed in NP40 lysis buffer [1% NP40, 20 mmol/L Tris pH 8.0, 137 mmol/L NaCl, 0.5 mmol/L EDTA, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 0.15 units/mL aprotinin, 20 μmol/L leupeptin, 1 mmol/L sodium vanadate] on ice for 20 minutes and cleared by centrifugation for 20 minutes at 14,000 × *g*. Protein content was measured by the Bio-Rad protein assay. One hundred micrograms of protein were resolved by SDS-PAGE, transferred, and detected with anti–phospho-EGFR (Tyr^{1068}), anti–phospho-AKT (Ser^{473}), or anti–phospho-p42/p44 MAPK antibody (Cell Signaling, Beverly, MA) using an ECL chemiluminescence system (Amersham Corp., Arlington Heights, IL). Corresponding membranes were reprobed with anti-EGFR (Santa Cruz, Santa Cruz, CA), anti-AKT (Cell Signaling) or anti-MAPK (Santa Cruz) antibody to ensure equal loading of protein. HER1 expression in cell lines was analyzed by Western blotting using the anti-EGFR antibody.

Cytotoxicity assay. To study the effect of EGFR inhibition by ZD1839 in neuroblastoma, A431, SY5Y, and NLF cells were seeded into 96-well plates at a density of 3×10^4 per well in 10% FBS-RPMI medium. We started with more cells for these experiments than in the cell viability assay to obtain an accurate reading by MTT after treatment. Different concentrations (0.1, 0.5, 1, 10, and 40 μmol/L) of ZD1839 were added after the cells were attached. The cells were treated for 72 hours and a colorimetric MTT assay was then done as detailed above. To study effects of the PI3K inhibitor LY294002 and the MAPK inhibitor U0126, cells were pretreated with LY294002 (20 μmol/L) or U0126 (10 μmol/L) after seeding, and then were treated with EGF (100 ng/mL) or EGF and concurrent ZD1839 (1 μmol/L) for 72 hours. An MTT assay was then preformed. The experiments were done in five replicates and each experiment was repeated thrice. Measurement of absorbance is used to represent cell number.

Statistical analysis. The results of the MTT assay were analyzed by paired Student's *t* test.

Results

HER1-4 expression in neuroblastoma cells. We found readily detectable *HER1* expression in 10 of 13 neuroblastoma cell lines examined by semiquantitative RT-PCR. The amount of *HER1* mRNA expressed was at least half of our internal control gene (*GAPDH*). The expression had no apparent correlation with the presence or absence of *MYCN* amplification. Most of the cell lines did not express a higher level of *HER1* than normal fetal brain. NLF, which had the highest expression, was comparable with the *HER1* level detected in the A431 cells, which have amplification of the *HER1* gene (Fig. 1*A*). Expression of HER1 protein in the neuroblastoma cell lines NLF, SKNSH, IMR5, SY5Y, and CHP134 was confirmed by Western blotting (Fig. 1*C*). NLF and SY5Y were

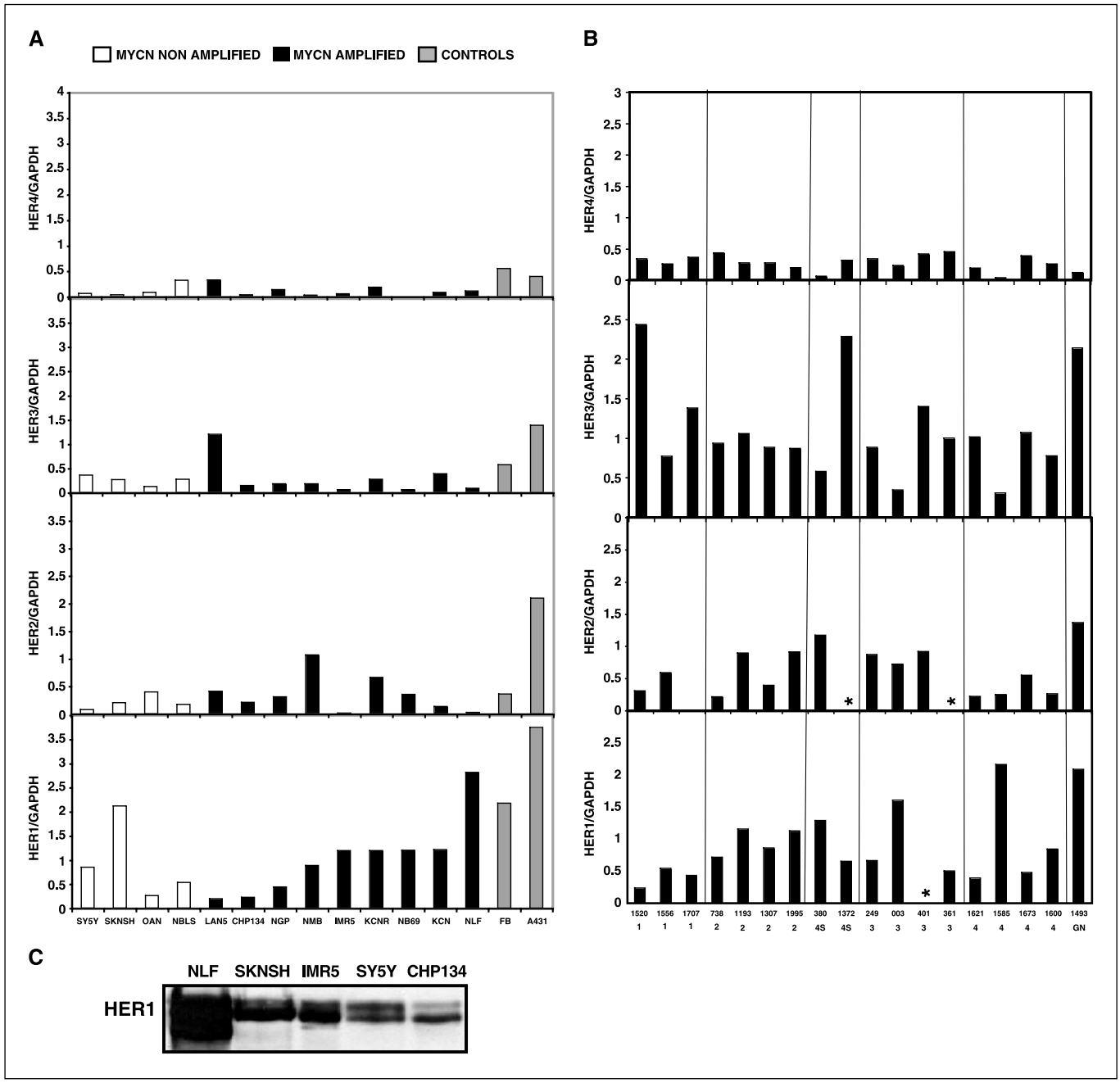

Figure 1. Expression of HER family receptors in neuroblastoma cell lines and tumors. *A* and *B,* mRNA expression of *HER* family receptors in neuroblastoma cell lines and primary tumors was detected by semiquantitative RT-PCR. The expression was quantified by densitometry. Ratio of the density of each target mRNA and its corresponding *GAPDH* was graphed. *A,* expression of *HER* family receptors in cell lines. *Open white columns,* cell lines without *MYCN* amplification. *Solid black columns, MYCN*-amplified cell lines. *Solid gray columns,* control cell line A431 and control tissue human fetal brain. *B,* expression of *HER* family receptors in primary tumors. *No data due to running out of tumor cDNA. *C,* protein expression of HER1 of some of the neuroblastoma cell lines. HER1 expression was detected by Western blotting.

representative of high and intermediate levels of HER1 expression, respectively, and they were used in further studies. The cell lines examined also expressed relatively low levels of *HER4* mRNA. *HER2* and *HER3* expression were generally low in the cell lines examined except for NMB (for *HER2*) and LAN-5 (for *HER3*), which expressed an mRNA level similar to the internal control gene *GAPDH* (Fig. 1*A*).

HER1 mRNA was also expressed in 18 of 18 primary neuroblastoma tumors examined, and the average *HER1* expression was similar to *GAPDH*. However, we did not find a significant correlation between expression level and tumor stage (Fig. 1*B*). We found readily detectable levels of *HER2* expression in 9 of 15 primary tumors. The primary tumors also expressed low levels of *HER4*, but *HER3* expression was generally higher. The expression level of the *HER* family genes was not significantly correlated with tumor stage (Fig. 1*B*). To confirm these findings, we did an analysis of *HER* family gene expression (by microarray analysis) in 101 additional primary tumors, representing carefully defined low,

intermediate, high, and ultrahigh risk groups. There was again no correlation between *HER* expression and either tumor stage or risk group (data not shown).

Epidermal growth factor enhances proliferation of some neuroblastoma cells. Because HER1 is expressed in neuroblastoma cells, we studied the effect of EGF exposure to determine if the receptor was functional. SY5Y has a moderate level of HER1 expression and NLF has a high level, comparable with the HER1-overexpressing cell line A431. Treatment of SY5Y cells with EGF (100 ng/mL) caused a 2-fold increase in cell number by day 7 ($P < 0.01$). Similar results were obtained in NLF cells (Fig. 2*A*). Neurite outgrowth was used as an indication of neuronal differentiation, but no outgrowth was observed during 7 days of EGF treatment. NLF cells have a faster growth rate than SY5Y cells under normal conditions. Other neuroblastoma cell lines, such as SKNSH, IMR5, and CHP134, were also screened for their response to EGF. We found that there was a general correlation between the level of EGFR expression and the magnitude of growth enhancement by EGF (data not shown).

To decide whether EGF increases cell number by increasing proliferation or decreasing apoptosis, we measured DNA content by flow cytometry. We found that treating cells with EGF resulted in a substantial decrease in the percentage of cells in the G_0-G_1 phase, with a corresponding increase in the percentage of cells in S and G_2-M phase. Pretreatment of cells with ZD1839 (1 μmol/L) prevents the cell cycle progression by EGF. The apoptosis fraction was very low for cells under all conditions, and there was no appreciable change in this fraction (Fig. 2*B*; Table 1). Similar results were seen in SY5Y cells, although to a lesser extent. Thus, EGF increases neuroblastoma cell number primarily by increasing cell proliferation.

ZD1839 blocks proliferation of neuroblastoma cells. ZD1839 is specific inhibitor of the EGFR tyrosine kinase at the micromole per liter range (22). Because EGF enhances the growth of some neuroblastoma cell lines, we investigated the effect of ZD1839 in neuroblastomas. A431 cells, which amplify and overexpress HER1, were sensitive to ZD1839 and used as a positive control. A431 cells, as well as the neuroblastoma cells SY5Y and NLF, were grown in

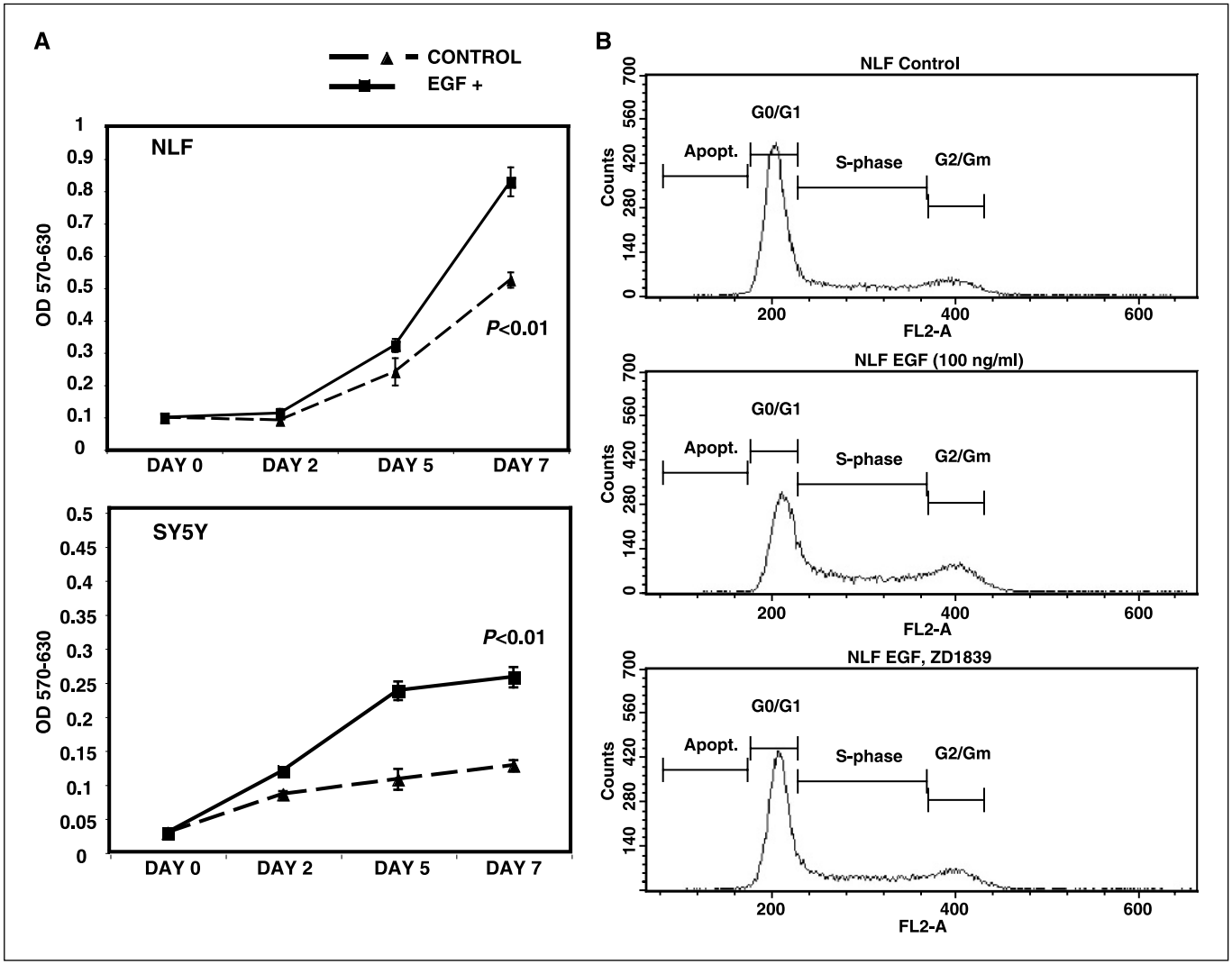

Figure 2. EGF enhances proliferation of NLF and SY5Y cell lines. *A,* cells were seeded in 24-well plates in 2% FBS-RPMI medium, and treated with EGF (100 ng/mL) for 2, 5, and 7 days. An MTT assay was done at indicated time points to measure cell number. *Points,* means of five replicates; *bars,* SD. The results shown are representative of three independent experiments. *B,* cells were seeded in 2% FBS without or with 100 ng/mL EGF or EGF plus ZD1839 (1 μmol/L). DNA content was measured by flow cytometry. Cell fit was done by the CELLQuest (version 5.1) program.

Table 1. Percentage of NLF cells in different phases of the cell cycle under different conditions

	Control	EGF	EGF + ZD1839
G_1-G_0 (%)	66.81	52.62	62.92
S (%)	20.04	26.72	22.00
G_2-M (%)	11.04	19.01	13.57
Apoptosis (%)	0.59	0.28	0.49

10% FBS and exposed to a range of concentrations of ZD1839 for 72 hours. Concentrations as low as 0.1 μmol/L for NLF cells and 0.5 μmol/L for SY5Y cells caused significant growth inhibition. At a concentration of 0.5 μmol/L, ZD1839 caused a 40% to 50% reduction of growth in both NLF and SY5Y cells by 72 hours. This concentration was similar to that needed for inhibition of the A431 cell line (data not shown), indicating that NLF and SY5Y were both very sensitive to ZD1839 (Fig. 3*A*). A similar concentration of ZD1839 was needed to inhibit EGF-stimulated growth in NLF cells to the same extent in 1% FBS (data not shown). Thus, the IC_{50} for ZD1839 was ~0.5 μmol/L for both NLF and SY5Y. At higher doses, ZD1839 also inhibits other kinase receptors, such as HER2, Flt-1, Raf, etc. (22). Thus, the growth inhibitory effect we observed at 10 to 40 μmol/L concentrations could be due to inhibition of other kinase receptors as well. Flow cytometry indicates that 1 μmol/L ZD1839 prevents EGF-mediated cell cycle progression from G_0-G_1 to the S and G_2 phases. Higher doses (10 μmol/L) of ZD1839 increases apoptosis as well (data not shown), probably due to simultaneous inhibition of other important cell signaling pathways.

ZD1839 blocks the effect of epidermal growth factor on neuroblastoma cells primarily through the phosphoinositide 3-kinase pathway. Phosphorylation of HER1 by EGF led to activation of the MAPK and PI3K pathways. These pathways are thought to be important for cell survival and proliferation (23, 24). EGF induces phosphorylation of AKT (Ser^{473}) and phosphorylation of both p42 and p44 MAPK (Figs. 3*B* and 4*B*, *first two lanes*).

We also examined the ability of ZD1839 to block downstream signaling pathways initiated by EGF. NLF and SY5Y cells were pretreated with different concentrations of ZD1839 before EGF treatment, and phosphorylation of HER1, AKT (Ser^{473}), and p42/p44 MAPK was examined. We found that ZD1839 greatly reduced phosphorylation of HER1 (Tyr^{1068}) at a concentration of 0.01 μmol/L. In NLF cells at a concentration of 1 μmol/L, ZD1839 markedly reduced phosphorylation of AKT (Ser^{473}). However, ZD1839 showed only moderate inhibition of the phosphorylation of MAPK at concentrations as high as 10 μmol/L (Fig. 3*B*). In SY5Y cells, inhibition of AKT phosphorylation (Ser^{473}) was evident at a concentration of 0.01 μmol/L, whereas inhibition of p42/p44 MAPK phosphorylation was not seen until 1 μmol/L. We also examined the effect of ZD1839 on two other neuroblastoma cell lines (NB69 and SKNSH), and both showed pathway inhibition similar to SY5Y. In summary, ZD1839 reduced the phosphorylation of HER1 at very low concentrations. It has a profound effect on PI3K/AKT pathway but a more modest effect on the phosphorylation of MAPK. Thus, the PI3K/AKT pathway seems to play a more important role in EGF-meditated cell proliferation.

To further support our conclusions regarding the relative importance of the PI3K/AKT and p42/p44 MAPK pathways, we studied the effect of LY294002 and U0126, which are specific inhibitors of these respective pathways. NLF and SY5Y cells grown in medium containing 2% FBS were pretreated with LY294002 or U0126 for 1 hour before EGF treatment. Twenty micromoles per liter of LY294002 significantly decreased cell number, whereas 10 μmol/L U0126 had no significant effect on either cell line (Fig. 4*A*, *white columns*). The same dose of LY294002 completely blocked EGF-mediated growth enhancement ($P < 0.01$) in both cell lines, whereas 10 μmol/L U0126 only modestly reduced EGF growth enhancement ($P > 0.01$) in SY5Y cells as determined by MTT assay.

Western blotting was done to ensure inhibition of specific pathways by corresponding inhibitors at the appropriate concentration. Twenty micromoles per liter of LY294002 almost completely blocked phosphorylation of AKT (Ser^{473}) induced by EGF specifically in SY5Y cells (Fig. 4*B*), whereas NLF needed up to 40 μmol/L LY294002 to completely block the pathway. Ten micromoles per liter U0126 completely blocked MAPK pathway in SY5Y cells, whereas as little as 1 μmol/L was effective in NLF cells. Thus, the effect of 10 μmol/L U0126 we saw in NLF (Fig. 4*A*) could be due to inhibition of other pathways. Given the fact that 1 μmol/L ZD1839 inhibited EGF-mediated growth enhancement and PI3K/AKT activation in NLF cells, but it has very little effect on MAPK phosphorylation (Fig. 3*B*), we conclude that the PI3K/AKT pathway is the primary pathway responsible for EGF-mediated proliferation.

Discussion

Neuroblastoma is a common pediatric cancer and a leading cause of morbidity and mortality in children. Currently, even very intensive, multimodality therapy has resulted in only modest improvement in the cure rate of the more aggressive neuroblastomas. Therefore, a better understanding of the genes, proteins, and pathways responsible for neuroblastoma tumorigenesis and progression may lead to the development of more effective, less toxic therapies.

We have shown that 10 of 13 neuroblastoma cell lines and all primary tumors tested expressed *HER1* at readily detectable levels. Other members of *HER* family receptors (*HER2, HER3*, and *HER4*) are also expressed in neuroblastoma cell lines at lower levels. *HER3* was expressed in primary neuroblastomas at levels similar to *HER1*. There was no correlation between tumor stage and expression of any of the *HER* receptors. This was further supported by data from a microarray analysis of HER expression in 101 neuroblastoma tumors (data not shown). Nevertheless, the EGF/HER1 pathway seems to be an important growth/proliferation pathway for neuroblastoma. The addition of exogenous EGF led to proliferation of SY5Y, NLF, and other neuroblastoma cells. Others have made similar observations on the NB69 neuroblastoma cell line (25). Because of the presence of other HER family receptors, HER1 may form a homodimer with itself or heterodimers with other members of this family. Other members of the HER family in neuroblastoma may be functional as well. For example, the HER3 receptor is expressed in the neuroblastoma cell line LAN-5 at readily detectable levels, and it can bind its ligand GGF-2 (neuregulin-2; ref. 26), as well as activate downstream PI3K/AKT and MAPK pathways (data not shown).

The HER1-specific tyrosine kinase inhibitor ZD1839 blocked the growth advantage induced by EGF. Furthermore, we examined downstream signaling pathways and showed that this specific inhibitor markedly reduced the EGF-induced autophosphorylation of HER1 and the phosphorylation of AKT (but not MAPK), suggesting that the EGF/HER1–mediated growth enhancement

effect is primarily mediated by the PI3K/AKT pathway. Furthermore, this inhibitory effect could be mimicked using LY249002, a specific inhibitor of PI3K/AKT pathway. However, treatment of cells with U0126, a specific inhibitor of p42/p44 MAPK, had only a modest effect, further supporting our conclusion that the primary growth enhancement effect of HER1 activation is mediated by the PI3K/AKT pathway.

Inhibition of tyrosine kinase receptors is an attractive approach for treating cancers for which a specific tyrosine kinase receptor pathway can be identified as important. ZD1839 (Iressa, gefitinib) has proven to be useful preclinically and clinically for treating EGFR-expressing tumors, such as non–small cell lung cancer, head and neck cancer, breast cancer, prostate cancer, and others; the general doses used clinically are 250 to 500 mg/d (27–30). Other EGFR inhibitors, such as Herceptin (trastuzumab), an anti-HER2 monoclonal antibody, have shown activity in a subset of breast cancers (31).

We have shown previously that members of the Trk family are expressed in both favorable and unfavorable neuroblastomas (2, 5, 7). TrkB and its ligand BDNF are expressed in the most aggressive, *MYCN*-amplified neuroblastomas, and this seems to represent an autocrine survival pathway for these tumors. The TrkB/BDNF pathway mediates survival and leads to activation of the PI3K/AKT pathway as well (1). CEP-751 is a Trk-specific tyrosine kinase inhibitor that is also under investigation in our laboratory. We have shown that CEP-751 inhibits growth of TrkB-expressing neuroblastoma cells *in vitro* and *in vivo* in mouse xenografts (32).

Here, we have shown that the EGF/HER1 pathway may also be an important growth/proliferation pathway for neuroblastomas, and low concentrations of ZD1839 inhibit growth of neuroblastoma cells. The IC_{50} of ZD1839 for NLF is 0.5 μmol/L in 10% FBS serum (and a similar dose under low serum conditions with EGF stimulation). This dose is comparable with that needed to inhibit the HER1-expressing A431 cell line in our study. Clinically, the

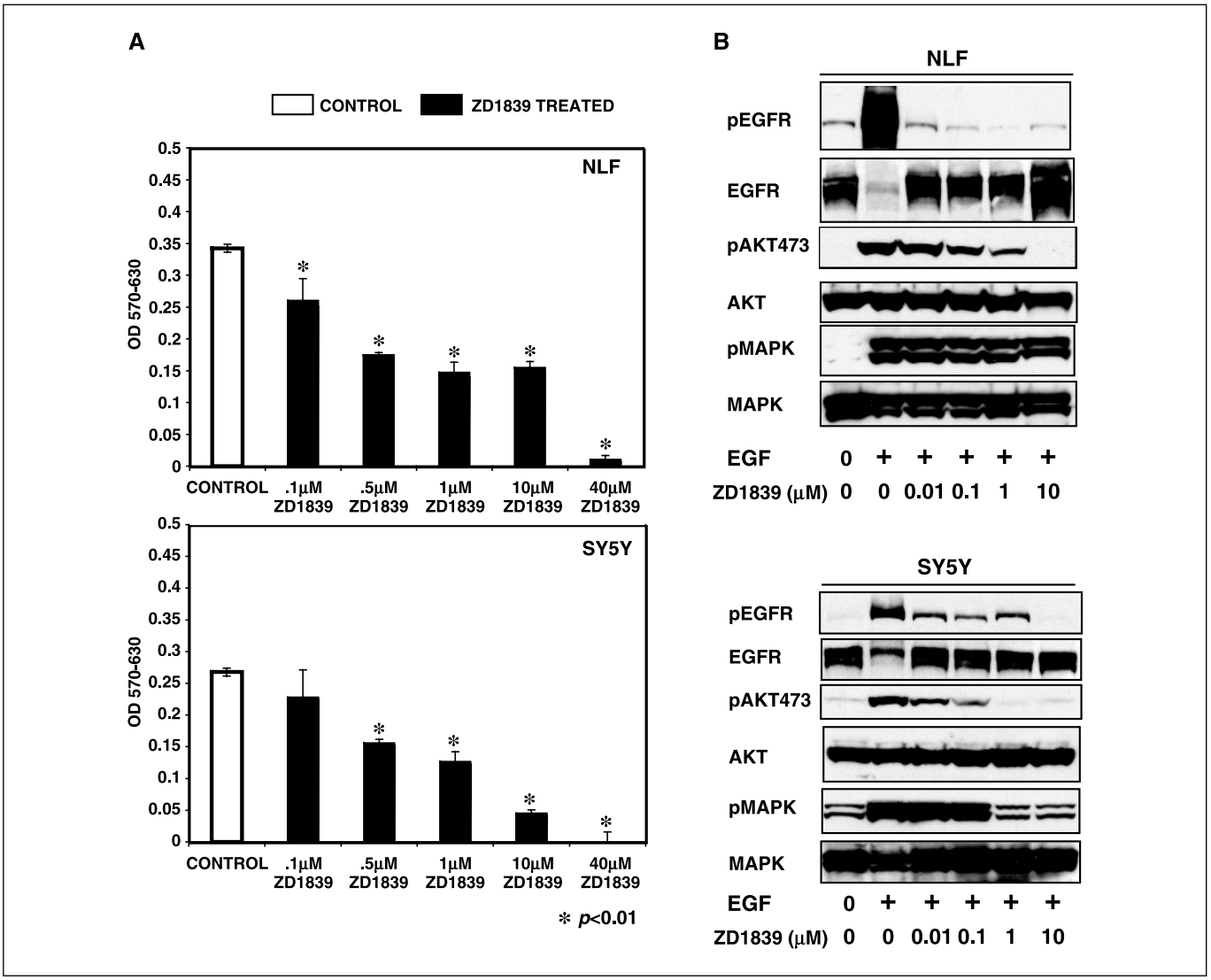

Figure 3. ZD1839 inhibits the growth of neuroblastoma cells. *A,* effect of ZD1839 on survival of NLF and SY5Y cells. Cells were treated with the indicated concentrations of ZD1839 for 72 hours and cell number was measured by MTT assay. ZD1839 significantly inhibits the proliferation of NLF cells at a concentration of 0.1 μmol/L and of SY5Y cells at 0.5 μmol/L ($P < 0.01$). *Columns,* mean of five replicates; *bars,* SD. *B,* cells were pretreated with ZD1839 for 1 hour before exposure to EGF for 5 minutes. Phosphorylation of EGFR (Tyr^{1068}), AKT (Ser^{473}), and MAPK was examined by Western blotting. The blots were stripped and reprobed with anti-EGFR, anti-AKT, and anti-MAPK antibody, respectively, to measure total protein loading in each lane.

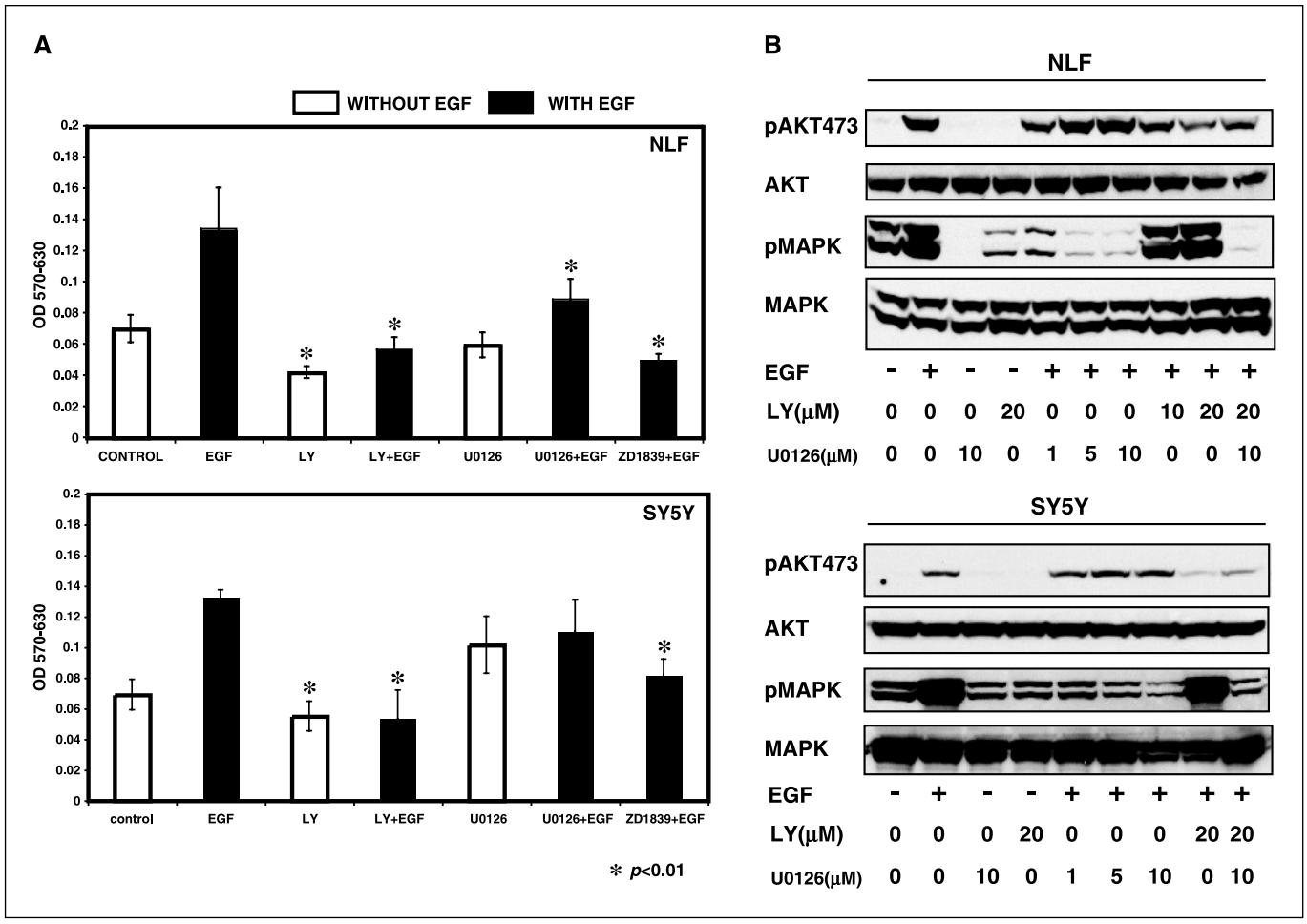

Figure 4. The PI3K/AKT specific inhibitor, LY294002, completely abolishes the proliferation effect of EGF. *A,* LY294002 completely blocks the effect of EGF. SY5Y and NLF cells were cultured in 2% serum medium in 96-well plates and pretreated with LY294002 (20 μmol/L) or U0126 (10 μmol/L) for 1 hour. EGF (100 ng/mL) or EGF plus ZD1839 (1 μmol/L) were added for 72 hours. Cell number was determined by MTT assay. LY294002 completely blocked EGF-mediated proliferation ($P < 0.01$), whereas 10 μmol/L U0126 only modestly reduced EGF growth enhancement; this is significant for NLF ($P < 0.01$) but not for SY5Y ($P = 0.12$). LY294002 alone significantly reduced cell number compared with control cells ($P < 0.01$), whereas U0126 alone did not have significant effect ($P > 0.01$). *Columns,* mean of five replicate samples; *bars,* SD. The results shown are representative of at least three independent experiments. *B*, Cells were pretreated with LY294002, U0126, or both for 30 minutes before being stimulated by EGF for 5 minutes. Phosphorylation of AKT (Ser^{473}) and MAPK was examined by Western blotting. Blots were stripped and reprobed with antibody against total AKT and MAPK to show equal protein loading in each lane.

geometric mean of plasma concentration is 0.3 to 1 mmol/L when patients were treated with a dose of 225 to 400 mg/d of ZD1839 (33, 34), which is more than serum-stimulated ZD1839 IC_{50} needed for NLF and SY5Y. Thus, ZD1839 seems promising as a novel agent for treating human neuroblastomas.

Other tyrosine kinase receptors may also facilitate the activity of EGFR or have an independent effect on growth or survival. Previous reports have shown that other receptors, such as insulin-like growth factor-IR (IGF-IR) and EPHR family receptors, are expressed in neuroblastomas (35, 36). IGF-IR activates AKT and MAPK pathways in SY5Y cells and leads to survival and differentiation (35). Interestingly, ligand binding of all of these tyrosine kinase receptors results in activation of similar intracellular signaling pathways, suggesting that activation of any of these growth factor/receptor pathways could lead to enhanced growth or survival. Deregulated tumor cell growth, especially for highly resistant tumor cells, could result from the overexpression of one or several tyrosine receptors.

A combination of currently available reagents inhibiting the HER family or Trk family receptors is an attractive, biologically based approach for the treatment of highly resistant neuroblastomas. In fact, the cooperative inhibitory effect of ZD1839 and trastuzumab has been shown on human breast cancer cell growth (37). We are currently investigating whether EGFR inhibition alone, or in combination with either Trk inhibition or conventional chemotherapeutic agents, may be a more effective and less toxic approach for treating human neuroblastoma xenografts or other mouse models of neuroblastoma.

Acknowledgments

Received 7/7/2004; revised 6/13/2005; accepted 8/17/2005.

Grant support: NIH grants CA-94194 and CA97323 (G.M. Brodeur, R. Ho, J.E. Minturn, and A.E. Evans), Young Investigator Award and Career Development Award from the American Society of Clinical Oncology (J.E. Minturn), Richard and Nancy Wolfson Young Investigator Award (J.E. Minturn and T. Hishiki), and Audrey E. Evans Endowed Chair (G.M. Brodeur).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank AstraZeneca for providing ZD1839 and Wing M. So for performing some confirmatory semiquantitative RT-PCR experiments.

References

1. Ho R, Eggert A, Hishiki T, et al. Resistance to chemotherapy mediated by TrkB in neuroblastomas. Cancer Res 2002;62:6462–6.
2. Nakagawara A, Arima-Nakagawara M, Scavarda NJ, Azar CG, Cantor AB, Brodeur GM. Association between high levels of expression of the Trk gene and favorable outcome in human neuroblastomas. N Engl J Med 1993; 328:847–54.
3. Suzuki T, Bogenmann E, Shimada H, Stram D, Seeger RC. Lack of high-affinity nerve growth factor receptors in aggressive neuroblastomas. J Natl Cancer Inst 1993; 85:377–84.
4. Kogner P, Barbany G, Dominici C, Castello MA, Raschella G, Persson H. Coexpression of messenger RNA for TRK protooncogene and low affinity nerve growth factor receptor in neuroblastoma with favorable prognosis. Cancer Res 1993;53:2044–50.
5. Nakagawara A, Azar CG, Scavarda NJ, Brodeur GM. Expression and function of TRK-B and BDNF in human neuroblastomas. Mol Cell Biol 1994;14:759–67.
6. Ryden M, Sehgal R, Dominici C, Schilling FH, Ibanez CF, Kogner P. Expression of mRNA for the neurotrophin receptor trkC in neuroblastomas with favourable tumour stage and good prognosis. Br J Cancer 1996; 74:773–9.
7. Yamashiro DJ, Nakagawara A, Ikegaki N, Liu XG, Brodeur GM. Expression of TrkC in favorable human neuroblastomas. Oncogene 1996;12:37–41.
8. Wells A. EGF receptor. Int J Biochem Cell Biol 1999; 31:637–43.
9. Olayioye MA, Graus-Porta D, Beerli RR, Rohrer J, Gay B, Hynes NE. ErbB-1 and ErbB-2 acquire distinct signaling properties dependent upon their dimerization partner. Mol Cell Biol 1998;18:5042–51.
10. Tang CK, Lippman ME. EGF family receptors and their ligands in human cancer. In: O'Malley BW, editor. Hormones and signaling. vol. I. San Diego (CA): Academic Press; 1998. p. 113–65.
11. Aaronson SA. Growth factors and cancer. Science 1991;254:1146–53.
12. Britsch S, Li L, Kirchhoff S, et al. The ErbB2 and ErbB3 receptors and their ligand, neuregulin-1, are essential for development of the sympathetic nervous system. Genes Dev 1998;12:1825–36.
13. Gilbertson RJ, Perry RH, Kelly PJ, Pearson AD, Lunec J. Prognostic significance of HER2 and HER4 coexpression in childhood medulloblastoma. Cancer Res 1997; 57:3272–80.
14. Gilbertson RJ, Bentley L, Hernan R, et al. ERBB receptor signaling promotes ependymoma cell proliferation and represents a potential novel therapeutic target for this disease. Clin Cancer Res 2002;8:3054–64.
15. Mischel PS, Cloughesy TF. Targeted molecular therapy of GBM. Brain Pathol 2003;13:52–61.
16. Janet T, Ludecke G, Otten U, Unsicker K. Heterogeneity of human neuroblastoma cell lines in their proliferative responses to basic FGF, NGF and EGF: correlation with expression of growth factors and growth factor receptors. J Neurosci Res 1995;40: 707–15.
17. Layfield LJ, Thompson JK, Dodge RK, Kerns BJ. Prognostic indicators for neuroblastoma: stage, grade, DNA ploidy, MIB-1-proliferation index, p53, HER-2/neu and EGFr—a survival study. J Surg Oncol 1995;59:21–7.
18. Bolufer P, Lluch A, Molina R, et al. Epidermal growth factor in human breast cancer, endometrial carcinoma and lung cancer. Its relationship to epidermal growth factor receptor, estradiol receptor and tumor TNM. Clin Chim Acta 1993;215:51–61.
19. Eggert A, Ikegaki N, Kwiatkowski J, Zhao H, Brodeur GM, Himelstein BP. High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas. Clin Cancer Res 2000;6:1900–8.
20. Merlino GT, Xu YH, Ishii S, et al. Amplification and enhanced expression of the *epidermal growth factor receptor* gene in A431 human carcinoma cells. Science 1984;224:417–9.
21. Eggert A, Brodeur GM, Ikegaki N. Relative quantitative RT-PCR protocol for TrkB expression in neuroblastoma using GAPD as an internal control. Biotechniques 2000;28:681–2, 6, 8–91.
22. Wakeling AE, Guy SP, Woodburn JR, et al. ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. Cancer Res 2002;62:5749–54.
23. Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. Exp Cell Res 2003;284:31–53.
24. Moghal N, Sternberg PW. Multiple positive and negative regulators of signaling by the EGF-receptor. Curr Opin Cell Biol 1999;11:190–6.
25. Murillo-Carretero M, Ruano MJ, Matarredona ER, Villalobo A, Estrada C. Antiproliferative effect of nitric oxide on epidermal growth factor-responsive human neuroblastoma cells. J Neurochem 2002;83:119–31.
26. Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. EMBO J 2000;19:3159–67.
27. Fukuoka M, Yano S, Giaccone G, et al. Multi-institutional randomized phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. J Clin Oncol 2003;21:2237–46.
28. Cohen EE, Rosen F, Stadler WM, et al. Phase II trial of ZD1839 in recurrent or metastatic squamous cell carcinoma of the head and neck. J Clin Oncol 2003; 21:1980–7.
29. Bundred NJ, Chan K, Anderson NG. Studies of epidermal growth factor receptor inhibition in breast cancer. Endocr Relat Cancer 2001;8:183–9.
30. Barton J, Blackledge G, Wakeling A. Growth factors and their receptors: new targets for prostate cancer therapy. Urology 2001;58:114–22.
31. Arteaga CL. Trastuzumab, an appropriate first-line single-agent therapy for HER2-overexpressing metastatic breast cancer. Breast Cancer Res 2003;5:96–100.
32. Evans AE, Kisselbach KD, Liu X, et al. Effect of CEP-751 (KT-6587) on neuroblastoma xenografts expressing TrkB. Med Pediatr Oncol 2001;36:181–4.
33. Baselga J, Rischin D, Ranson M, et al. Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. J Clin Oncol 2002;20:4292–302.
34. Albanell J, Rojo F, Baselga J. Pharmacodynamic studies with the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839. Semin Oncol 2001; 28:56–66.
35. Kurihara S, Hakuno F, Takahashi S. Insulin-like growth factor-I-dependent signal transduction pathways leading to the induction of cell growth and differentiation of human neuroblastoma cell line SH-SY5Y: the roles of MAP kinase pathway and PI3-kinase pathway. Endocr J 2000;47:739–51.
36. Tang XX, Zhao H, Robinson ME, et al. Implications of EPHB6, EFNB2, and EFNB3 expressions in human neuroblastoma. Proc Natl Acad Sci U S A 2000; 97:10936–41.
37. Normanno N, Campiglio M, De LA, et al. Cooperative inhibitory effect of ZD1839 (Iressa) in combination with trastuzumab (Herceptin) on human breast cancer cell growth. Ann Oncol 2002;13:65–72.