

# An inhibitor of the EGF receptor family blocks myeloma cell growth factor activity of HB-EGF and potentiates dexamethasone or anti-IL-6 antibody-induced apoptosis

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We previously found that some myeloma cell lines express the heparin-binding epidermal growth factor–like growth factor (*HB-EGF*) gene. As the proteoglycan syndecan-1 is an HB-EGF coreceptor as well as a hallmark of plasma cell differentiation and a marker of myeloma cells, we studied the role of HB-EGF on myeloma cell growth. The *HB-EGF* gene was expressed by bone marrow mononuclear cells in 8 of 8 patients with myeloma, particularly by monocytes and stromal cells, but not by purified primary myeloma cells. Six of 9 myeloma cell lines

and 9 of 9 purified primary myeloma cells expressed *ErbB1* or *ErbB4* genes coding for HB-EGF receptor. In the presence of a low interleukin-6 (IL-6) concentration, HB-EGF stimulated the proliferation of the 6 *ErbB1*<sup>+</sup> or *ErbB4*<sup>+</sup> cell lines, through the phosphatidylinositol 3-kinase/AKT (PI-3K/AKT) pathway. A pan-ErbB inhibitor blocked the myeloma cell growth factor activity and the signaling induced by HB-EGF. This inhibitor induced apoptosis of patients' myeloma cells cultured with their tumor environment. It also increased patients' myeloma cell apo-

ptosis induced by an anti-IL-6 antibody or dexamethasone. The ErbB inhibitor had no effect on the interaction between multiple myeloma cells and stromal cells. It was not toxic for nonmyeloma cells present in patients' bone marrow cultures or for the growth of hematopoietic progenitors. Altogether, these data identify ErbB receptors as putative therapeutic targets in multiple myeloma. (*Blood*. 2004;103:1829-1837)

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## Introduction

Multiple myeloma (MM) is a B-cell neoplasia characterized by the accumulation of clonal malignant plasma cells in the bone marrow. In most patients, malignant plasma cells require mediators delivered by the tumor environment to survive and proliferate. Interleukin-6 (IL-6) is one of these mediators produced in the bone marrow environment.<sup>1,2</sup> However, IL-6 alone can support the growth of myeloma cells in vitro only in patients with extramedullary proliferation.<sup>3</sup> In patients with intramedullary MM, additional factors produced by the tumor environment are necessary to promote the survival of primary myeloma cells, together with IL-6.<sup>3</sup> Searching for such intercellular communication signals with DNA arrays, we found that some myeloma cell lines expressed the heparin-binding epidermal growth factor–like growth factor (*HB-EGF*) gene<sup>4</sup> and that autocrine HB-EGF was involved in the IL-6–induced survival of 2 myeloma cell lines.<sup>5</sup>

HB-EGF is one of the 11 members of the epidermal growth factor (EGF)–related peptide growth factor family. This family binds to and induces the homo- or heterodimerization of 3 receptors: the EGF receptor *ErbB1*, *ErbB3*, and *ErbB4*. *ErbB2* is a fourth member unable to bind EGF ligands. *ErbB2* is a preferred heterodimerization partner for all other ErbB members and increases ErbB receptor signaling.<sup>6</sup> The EGF family includes EGF, transforming growth factor  $\alpha$ , amphiregulin, and epigen, which bind to *ErbB1*, and HB-EGF, betacellulin, and epiregulin, which bind to both *ErbB1* and *ErbB4*.<sup>7</sup> It also includes the 4 neuregulins. Neuregulin 1 and 2 bind to both

*ErbB3* and *ErbB4* and neuregulin 3 and 4 bind only to *ErbB4*.<sup>8</sup> ErbB receptors can cross-communicate with other receptors, including growth hormone receptor,<sup>9</sup> G-protein–coupled receptors,<sup>10</sup> gp130 IL-6 transducer,<sup>11,12</sup> or IGF-1 receptor.<sup>13,14</sup> Recently, interferon  $\alpha$  (IFN- $\alpha$ ) was shown to trigger *ErbB3* phosphorylation in the KAS-6/1 myeloma cell line.<sup>15</sup>

An abnormal expression of ErbB receptors and/or EGF family ligands has been reported in numerous cancers.<sup>16–18</sup> In particular, *ErbB1* is expressed or overexpressed in a wide variety of solid human tumors, including non–small cell lung (NSCL), prostate, breast, colorectal, and ovarian cancers.<sup>19</sup> The *ErbB4* gene is also amplified in cancer and is associated with a poor prognosis.<sup>20</sup> The *ErbB2* gene is frequently amplified in epithelial cancers, mainly melanoma, breast, and ovarian cancers, leading to abnormal EGF signaling.<sup>21</sup> Expression of *ErbB3* is seen in many of the same tumor types that overexpress *ErbB2*.<sup>22</sup> The involvement of the EGF/ErbB family in cancer has encouraged the development of 2 families of ErbB inhibitors in clinical studies: monoclonal antibodies and ErbB tyrosine kinase inhibitors. ErbB kinase inhibitors are small molecules that compete with adenosine triphosphate binding to the ErbB kinase domains. They block ErbB kinase activity specifically, but not the activity of other receptors.<sup>23</sup> Antibodies to *ErbB2* are currently used in the treatment of breast and ovarian cancers. Antibodies to *ErbB1* and inhibitors of ErbB kinase activity are under active investigation in phase 1 to phase 3 trials for a variety of tumors.<sup>24</sup>

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In this study, we show that HB-EGF was a growth factor for 6 of 9 myeloma cell lines that expressed *ErbB1* and/or *ErbB4* HB-EGF receptors, but not for 3 nonresponsive cell lines. HB-EGF activity required a low concentration of IL-6 and was blocked by an anti-IL-6 antibody. HB-EGF triggered the PI-3K/AKT pathway, but not the Janus kinase–signal transducer and activator of transcription (JAK/STAT) and mitogen-activated protein kinase (MAPK) pathways. The biologic activity of HB-EGF was blocked by PD169540, a pan-ErbB kinase inhibitor analog to the CI-1033 clinical-grade inhibitor.<sup>25</sup> PD169540 ErbB inhibitor induced primary myeloma cells apoptosis in short-term culture and strongly potentiated dexamethasone (DEX) or anti-IL-6 monoclonal antibody (MoAb)-induced apoptosis. Altogether, these data provide a framework for targeting the ErbB pathway in novel, biologically based therapeutics in multiple myeloma.

## Materials and methods

### Myeloma cell lines and reagents

The human myeloma cell lines (HMCLs) were obtained in our laboratory and their characteristics have been reported previously.<sup>4,26,27</sup> Eight are IL-6–dependent myeloma cell lines (XG-1, XG-2, XG-5, XG-6, XG-7, XG-11, XG-16, XG-19). Their growth is dependent on addition of exogenous IL-6. Upon removal of IL-6, myeloma cells progressively apoptose.<sup>3,28</sup> One is autonomously growing (RPMI 8226). The HMCLs were routinely maintained in RPMI 1640, 10% fetal calf serum (FCS) and 2 ng/mL IL-6. The experiments were performed in RPMI 1640 with 5% FCS.

Bone marrow or peripheral blood samples were obtained from patients with intramedullary myeloma or with plasma cell leukemia (PCL), after they had provided informed consent. Recombinant HB-EGF and IL-6 were purchased from R&D Systems (Minneapolis, MN), the PD153035 and AG1478 ErbB1 inhibitors from Alexis Biochemicals (San Diego, CA), the LY294002 PI-3 kinase inhibitor from Sigma (St Louis, MO), the PD169540 pan-ErbB kinase inhibitor was a generous gift from Pfizer Global Research and Development (Ann Arbor, MI), and the B-E8 anti-IL-6 MoAb was a generous gift from Dr Wijdenes (Diacione, Besancon, France).

### Cell proliferation assay

Cells were cultured for 5 to 7 days (depending on the cell lines) in 96-well flat-bottomed microtiter plates at  $10^4$  cells/well in 200  $\mu$ L RPMI 1640 culture medium and 5% FCS. Various concentrations of cytokines or growth factors or inhibitors of cytokine/growth factor were added at the beginning of the culture in 6 culture wells per group. At the end of the culture, cells were pulsed with tritiated thymidine (Amersham Pharmacia Biotech, Orsay, France) for 12 hours, then harvested and counted as reported previously.<sup>29</sup>

### Detection of apoptotic cells

Myeloma cells were cultured for 3 days in 24-well flat-bottomed microtiter plates at  $10^5$  cells/well in 1 mL RPMI 1640 medium with 5% FCS and various cytokines/growth factors or inhibitors. At the end of the culture, cells were washed once in phosphate-buffered saline (PBS) and suspended in annexin V–fluorescein isothiocyanate (FITC) solution (Boehringer, Mannheim, Germany). Fluorescence was analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

### Reverse transcriptase–polymerase chain reaction (RT-PCR)

We generated cDNA with 2  $\mu$ g total RNA using the Superscript II reverse transcriptase (Life Technologies) and oligo d(T)<sub>12-18</sub> (Amersham Pharmacia Biotech) as primer. Each 25- $\mu$ L PCR reaction contained 1  $\mu$ L of the first strand cDNA, 1  $\mu$ M of each primer (sense and antisense) designed for

annealing to different exons, 0.2 mM of each dNTP, 1.5 mM MgCl<sub>2</sub>, 1X polymerase buffer, and 2 units of *Taq* polymerase (Life Technologies). The following primers were used: HB-EGF, 5'-TGG TGC TGA AGC TCT TTC TGG (sense) and 5'-GTG GGA ATT AGT CAT GCC CAA (antisense);  $\beta$  actin, 5'-TCC CTG GAG AAG AGC TAC GA (sense) and 5'-AGC ACT GTG TTG GCG TAC AG (antisense); ErbB1, 5'-CAG CGC TAC CTT GTC ATT CAG (sense) and 5'-TCA TAC TAT CCT CCG TGG TCA (antisense); ErbB2, 5'-CGC TTT GTG GTC ATC CAG AAT G (sense) and 5'-TCG TGT TCA CAC TGG CAC GTC (antisense); ErbB3, 5'-TGG CCC GAG ACC CAC CAC GGT ATC TG (sense) and 5'-AGT TAC GTT CTC TGG GCA TTA GCC TT (antisense); ErbB4, 5'-AGT TTT CAA GGA TGG CTC GAG ACC CTC (sense) and 5'-AGC TTA CAC CAC AGT ATT CCG GTG TCT (antisense).

The sizes of the PCR products were as follows: HB-EGF, 605 bp;  $\beta$  actin, 194 bp; ErbB1, 726 bp; ErbB2, 821 bp; ErbB3, 1129 bp; and ErbB4, 1013 bp. The amplification profile was 1 minute at 95°C, 1 minute at 62°C (HB-EGF) or 60°C ( $\beta$  actin, ErbB1, ErbB2, ErbB3, and ErbB4), 1 minute at 72°C, followed by a final extension of 10 minutes at 72°C. The cycle number was 25 for  $\beta$  actin and 30 for HB-EGF, ErbB1, ErbB2, ErbB3, and ErbB4. Reaction products were electrophoresed on a 1.3% agarose gel.

### Purification of primary myeloma cells and CD14 monocytes

Malignant plasma cells were purified (>95% myeloma cells) from 9 patients with MM (5 with intramedullary myeloma and 4 with plasma cell leukemia), with the M115 anti-CD138 MoAb and antimouse IgG1 MACS microbeads (Miltenyi Biotech, Paris, France) as described.<sup>30</sup> Bone marrow CD14 monocytes were purified (>98% CD14 cells) from the bone marrow of 3 patients with MM with anti-CD14 MACS microbeads (Miltenyi Biotech).

### Bone marrow stromal cell (BMSC) culture

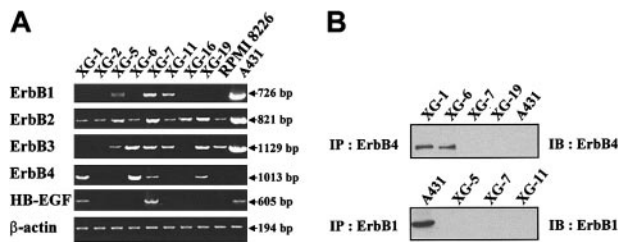
BMSCs were obtained from 5 patients with MM. Bone marrow mononuclear cells were obtained after Ficoll-Hypaque density centrifugation and cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 20% FCS at a concentration of  $10^6$  cells/mL. After 3 days, nonadherent cells were removed and fresh culture medium with 20% FCS was added. The culture medium was substituted with fresh medium and 20% FCS once a week. When a confluent layer of adherent cells was obtained, the cells were trypsinized and cultured in DMEM supplemented with 10% FCS.

### Adhesion assay of myeloma cells to BMSCs and IL-6 production

BMSCs obtained from one patient were immortalized with a human telomerase reverse transcriptase gene–green fluorescent protein (hTERT-GFP) retrovirus produced in our laboratory. After selection with G418, 100% of the stromal cells expressed GFP as assayed with fluorescence-activated cell sorting (FACS). Stromal cells were trypsinized and plated in 24-well culture plates at a density of  $2.5 \times 10^4$  cells/well and incubated overnight at 37°C with or without the PD169540 pan-ErbB inhibitor. XG-11 myeloma cells ( $1.5 \times 10^5$  per well) were added and cultured for 24 hours. Nonadherent cells were counted and adherent cells were trypsinized and counted. In the 2 cell populations, GFP<sup>+</sup> stromal cells were quantified using FACS analysis. The IL-6 production by BMSCs after myeloma cell adhesion was assayed as described.<sup>31</sup> Briefly, both confluent BMSCs and myeloma cells were preincubated with or without PD169540. Myeloma cells and BMSCs were cocultured for 24 hours at 37°C, with or without PD169540, in 4 different culture wells per group. The culture supernatant was recovered at the end of the culture and assayed for IL-6 with an enzyme-linked immunosorbent assay (ELISA; Beckman-Coulter, Mar-seilles, France).

### Immunoprecipitation

Myeloma cells ( $20 \times 10^6$ ) were lysed in Tris, pH 7.4, 50 mM, NaCl 150 mM, 5 mM EDTA (ethylenediaminetetraacetic acid), 10 mM sodium pyrophosphate (NaPPi), 1 mM Na<sub>3</sub>VO<sub>4</sub>, 160 mM NaF, 2.5 mM PMSF (phenylmethylsulfonyl fluoride), 10  $\mu$ M leupeptin, 2  $\mu$ M pepstatin, 10



**Figure 1. Expression of EGF receptor family members in myeloma cell lines.** (A) *ErbB1*, *ErbB2*, *ErbB3*, *ErbB4*, and *HB-EGF* gene expression was assayed with RT-PCR in 9 myeloma cell lines. The A431 epidermoid carcinoma cell line was used as a control. Amplification of  $\beta$  actin shows the equivalence of the cDNA loading and amplification. Results are from one experiment representative of 3. (B) Myeloma cells or A431 cells were lysed and the lysates were immunoprecipitated with the H4.72.8 anti-ErbB4 or the LA-22 anti-ErbB1 antibody. The precipitated material was separated on a 7.5% SDS-PAGE and analyzed by Western blotting with a second anti-ErbB antibody (anti-ErbB4, clone C-18, or anti-ErbB1, clone LA-1). Results are of one experiment representative of 3.

mg/L aprotinin, 1% NP 40, 5 g/L deoxycholate. The lysates were immunoprecipitated with an anti-ErbB4 (clone H4.72.8; Neomarkers, Union City, CA) or anti-ErbB1 (clone LA-22; Upstate Biotechnology, Lake Placid, NY) antibody. The precipitated material was separated on a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting with an anti-ErbB4 (clone C-18; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-ErbB1 antibody (clone LA-1; Upstate Biotechnology).

**Western blot**

Myeloma cells were starved overnight in RPMI 1640 culture medium in the presence of 1% bovine serum albumin (BSA), without cytokines. Cells were then stimulated with HB-EGF (5  $\mu$ g/mL), insulin-like growth factor 1 (IGF-1; 1  $\mu$ g/mL), or IL-6 (20 ng/mL) for 15 minutes at 37°C. Cells were lysed in 10 mM Tris-HCl (pH 7.05), 50 mM NaCl, 50 mM NaF, 30 mM NaPPi, 1% Triton X-100, 5  $\mu$ M ZnCl<sub>2</sub>, 100  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>, 1 mM dithiothreitol (DTT), 20 mM  $\beta$ -glycerophosphate, 20 mM p-nitrophenolphosphate (PNPP), 20  $\mu$ g/mL aprotinin, 2.5  $\mu$ g/mL leupeptin, 0.5 mM PMSF, 0.5 mM benzamidine, 5  $\mu$ g/mL pepstatin, and 50 nM okadaic acid. Lysates were resolved in 7.5% SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher and Schuell, Kassel, Germany). Membranes were blocked for 2 hours at room temperature in 140 mM NaCl, 3 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.1% tween 20 (TBS-T), 5% nonfat milk, and then immunoblotted with the primary antibodies: anti-phospho-STAT3, anti-phospho-AKT, and anti-phospho-MAPK rabbit polyclonal antibodies from New England Biolabs (Beverly, MA), at a 1:1000 dilution. As a control for protein loading, we used anti-STAT3 mouse monoclonal antibody from Transduction Laboratories (Lexington, KY), anti-AKT rabbit polyclonal antibody from New England Biolabs, and anti-MAPK rabbit polyclonal antibody from Santa Cruz Biotechnology. The primary antibodies were visualized with goat antirabbit (Sigma) or goat antimouse (Biorad, Hercules, CA) peroxidase-conjugated antibodies by an enhanced chemiluminescence detection system.

**Mononuclear cell culture**

Mononuclear cells from 8 patients with MM were cultured for 5 days at 5  $\times$  10<sup>5</sup> cells/mL in RPMI 1640 medium, 5% FCS, 1 ng/mL IL-6, without (control) or with the PD169540 pan-ErbB inhibitor (1  $\mu$ M), the B-E8 anti-IL-6 MoAb (10  $\mu$ g/mL) or 10<sup>-6</sup> M dexamethasone (DEX) used alone or in combination. In each culture group, the viability and cell counts were assayed and myeloma cells were stained with an anti-CD138-PE MoAb (Beckman-Coulter).

**Assay of hematopoietic colony-forming cells**

Bone marrow mononuclear cells from 5 patients with MM were grown in a semisolid culture medium with hematopoietic cytokines (GF

H4434; StemCell Technologies, Vancouver, BC, Canada). Cells were cultured without (control) or with the PD169540 pan-ErbB kinase inhibitor (1  $\mu$ M), the B-E8 anti-IL-6 MoAb (10  $\mu$ g/mL), or 10<sup>-6</sup> M DEX used alone or in combination. The number of granulocyte-macrophage colonies was counted on day 14 of incubation.

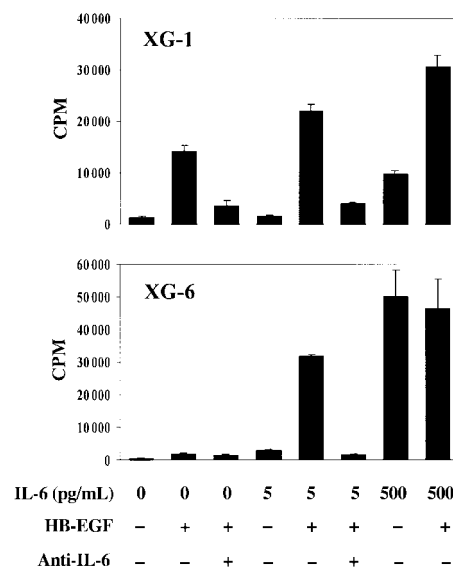
**Statistical analysis**

Results were compared with a Student *t* test or a Wilcoxon test for pairs.

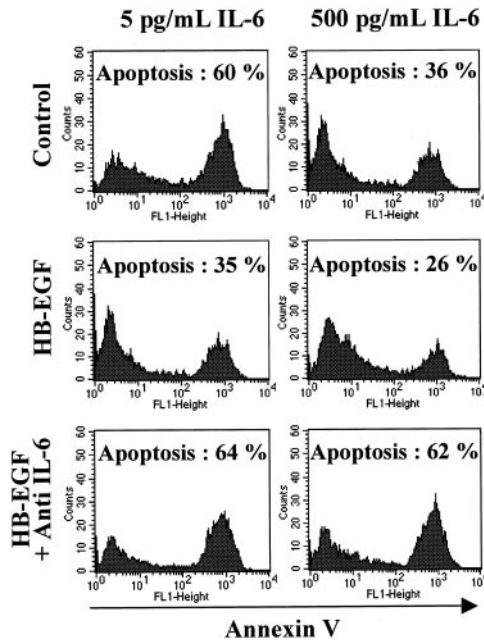
**Results**

**Gene expression of EGF receptor family members and HB-EGF in myeloma cell lines**

Gene expression of the 4 members of the EGF receptor family was studied in 9 myeloma cell lines. Eight are IL-6-dependent myeloma cell lines (XG-1, XG-2, XG-5, XG-6, XG-7, XG-11, XG-16, XG-19) and one is an autonomously growing cell line (RPMI 8226). The A431 epidermoid carcinoma cell line was used as a control. In agreement with previous studies,<sup>32</sup> a high *ErbB1*, *ErbB2*, and *ErbB3* expression, unlike *ErbB4*, was found in A431 cells (Figure 1A). *ErbB2* was expressed in all cell lines and *ErbB3* in 6 of 9 cell lines. The gene expression was always weaker than that found in the A431 cells. Three myeloma cell lines (XG-5, XG-7, and XG-11) expressed *ErbB1*. Four of 9 myeloma cell lines expressed *ErbB4*, with a high expression in XG-1 and XG-6 cells and a weak one in XG-7 and XG-19 cells. ErbB4 protein was detected by immunoprecipitation in XG-1 and XG-6 cells and was probably too low to be detected in XG-7 and XG-19 cells (Figure 1B). ErbB1 was detected by immunoprecipitation in A431, but protein was below the detection limit in XG-5, XG-7, and XG-11 cells (Figure 1B). In agreement with our previous report,<sup>4</sup> we found *HB-EGF* expression in 2 of 9 myeloma cell lines (Figure 1A).



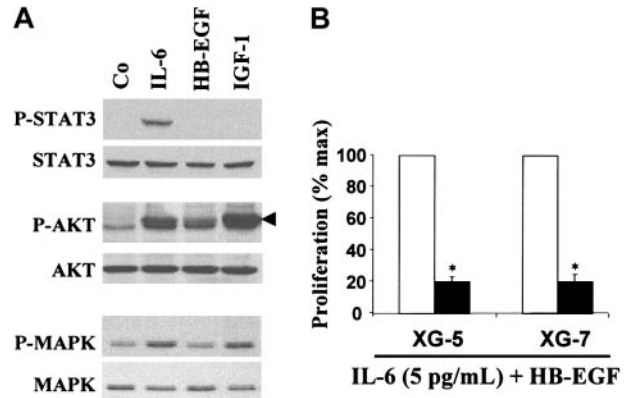
**Figure 2. Sensitivity of myeloma cell lines to HB-EGF.** XG-1 and XG-6 cells were IL-6 starved for 3 hours and cultured in RPMI 1640 culture medium and 5% FCS with either 5 pg/mL IL-6, or 5 pg/mL IL-6 and 1  $\mu$ g/mL HB-EGF, or 500 pg/mL IL-6, or 500 pg/mL IL-6 and 1  $\mu$ g/mL HB-EGF (with or without 10  $\mu$ g/mL B-E8 anti-IL-6 MoAb). Cells were cultured for 6 days and were pulsed for 12 hours with tritiated thymidine at the end of the culture. Data are means  $\pm$  SDs of the tritiated thymidine incorporation determined on sixuplicate culture wells and are those from one experiment representative of 4.



**Figure 3. HB-EGF reduces the apoptosis induced by IL-6 removal.** XG-1 cells were IL-6 starved for 3 hours and cultured in RPMI 1640 culture medium and 5% FCS with either 5 pg/mL IL-6, or 5 pg/mL IL-6 and 1 μg/mL HB-EGF, or 500 pg/mL IL-6, or 500 pg/mL IL-6 and 1 μg/mL HB-EGF (with or without 10 μg/mL B-E8 anti-IL-6 MoAb). Cells were cultured for 3 days and stained with FITC-annexin V to determine the percentage of apoptotic cells. Results are from one experiment representative of 3.

**Sensitivity of myeloma cell lines to HB-EGF**

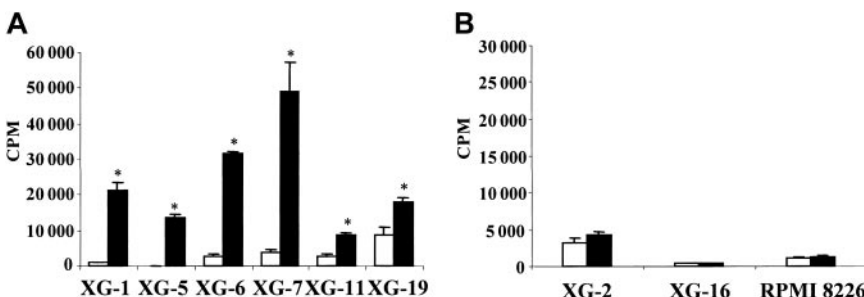
To investigate the effect of HB-EGF on myeloma cell growth, we first used the XG-1 and XG-6 cell lines, which cannot survive and proliferate without adding IL-6 when cultured at a low cell density.<sup>26</sup> HB-EGF induced the proliferation of XG-1 cells and had no effect on XG-6 cells (Figure 2). One difference between XG-1 and XG-6 cells is a low production of autocrine IL-6 by XG-1 cells.<sup>33</sup> This production of autocrine IL-6 by XG-1 cells is too weak to support the growth of XG-1 cells alone but contributed to the HB-EGF-induced proliferation since it was abrogated by anti-IL-6 MoAb (Figure 2). In the presence of a low IL-6 concentration (5 pg/mL), HB-EGF dramatically increased the IL-6-induced proliferation of XG-1 and XG-6 myeloma cells 14- and 12-fold, respectively. Anti-IL-6 MoAb abrogated this effect (Figure 2). With a high IL-6 concentration, HB-EGF increased the proliferation of XG-1 cells 3-fold and did not increase that of XG-6 cells, which was likely already at its maximum level. A significant effect was found with 100 ng/mL HB-EGF and a maximum effect with 100 ng/mL to 1000 ng/mL, depending on the myeloma cell lines (results not shown). Using annexin V, we observed that HB-EGF protected myeloma cells from apoptosis (Figure 3). Again, the myeloma cell survival activity of HB-EGF required a low concentration of IL-6 and was inhibited by



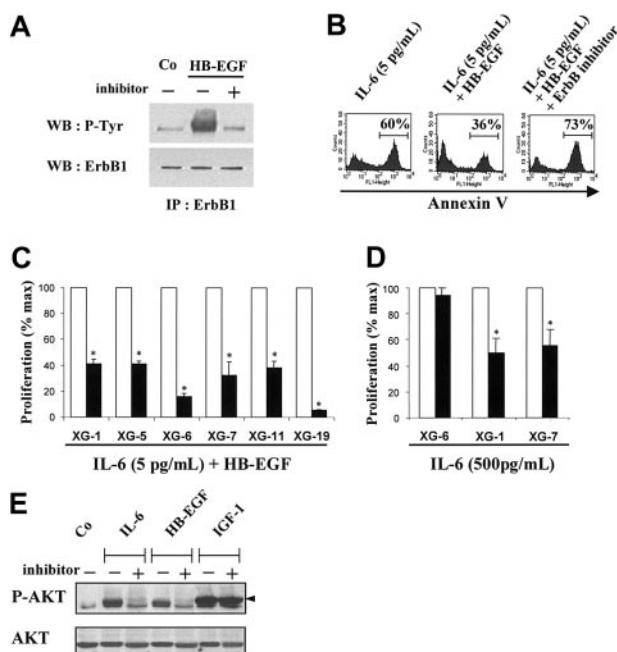
**Figure 5. HB-EGF activity is mediated by activation of the PI-3K/AKT pathway.** (A) XG-7 myeloma cells were starved overnight in culture medium without serum or cytokines. Cells were then stimulated with either IL-6 (20 ng/mL), IGF-1 (1 μg/mL), or HB-EGF (5 μg/mL) for 15 minutes at 37°C. Lysates from unstimulated (Co) or stimulated myeloma cells were immunoblotted with anti-phospho-specific STAT3 antibody (top panel) and then reprobbed with anti-STAT3 antibody (second panel), anti-phospho-specific AKT antibody (third panel) and reprobbed with anti-AKT antibody (fourth panel), anti-phospho-specific MAPK antibody (fifth panel) and reprobbed with anti-MAPK antibody (bottom panel). (B) XG-5 and XG-7 myeloma cell lines were IL-6 starved for 3 hours and cultured in RPMI 1640 culture medium and 5% FCS with 5 pg/mL IL-6 and 1 μg/mL HB-EGF without (□) or with (■) the PI-3K inhibitor LY294002 (25 μM). Cells were cultured for 6 days and pulsed with tritiated thymidine at the end of the culture. Mean tritiated thymidine incorporations were determined in sixplicate culture wells and results are expressed as percentages ± SDs of the mean proliferation without ErbB inhibitor. \*Indicates that the mean value is statistically significantly different from that obtained with 5 pg/mL IL-6 and HB-EGF without inhibitor, using a Student *t* test (*P* ≤ .05).

adding an anti-IL-6 antibody. Results obtained with XG-1 and XG-6 cells were extended to the 6 IL-6-dependent myeloma cell lines that expressed the *ErbB1* or *ErbB4* gene. Their proliferation was highly stimulated by adding HB-EGF (Figure 4A). No effect was found in the other 3 cell lines, which failed to express *ErbB1* or *ErbB4* (Figure 4B). These data indicate that HB-EGF enhances survival and proliferation of IL-6-dependent MM cell lines.

We next looked for signaling molecules activated by HB-EGF. We used the XG-7 myeloma cell line that is the most sensitive to HB-EGF (Figure 4A). HB-EGF induced AKT phosphorylation but not STAT3 phosphorylation (Figure 5A, lane 3). HB-EGF, used alone or with a small amount of IL-6, did not induce MAPK phosphorylation in myeloma cells after a short exposure (15 minutes; Figure 5A, lane 3) or longer exposures (60 minutes, 2 hours, 24 hours; results not shown). As a control, we used IL-6, which activated the JAK/STAT, MAPK, and PI3-K/AKT pathways (Figure 5A, lane 2), and IGF-1, which activated the MAPK and PI3-K/AKT pathways (Figure 5A, lane 4), in agreement with previous reports.<sup>29,34,35</sup> Indeed, the PI-3K/AKT pathway was critical for HB-EGF-induced myeloma cell proliferation since the PI-3K inhibitor LY294002 inhibited this proliferation (Figure 5B).



**Figure 4. Correlation between ErbB1/ErbB4 expression and HB-EGF activity on myeloma cells.** *ErbB1/ErbB4*-positive myeloma cells (A) and *ErbB1/ErbB4*-negative myeloma cells (B) were cultured for 5 to 7 days in RPMI 1640, 5% FCS, and 5 pg/mL IL-6, without (□) or with (■) HB-EGF and then pulsed with tritiated thymidine for 12 hours at the end of the culture. Data are expressed as means ± SDs of tritiated thymidine incorporation determined on sixplicate culture well. \*Indicates that the mean value is statistically significantly different from that obtained with 5 pg/mL IL-6 alone, using a Student *t* test (*P* ≤ .05).



**Figure 6. The myeloma cell growth factor activity of HB-EGF is inhibited by a pan-ErbB kinase inhibitor.** (A) A431 cells were stimulated with HB-EGF, with or without PD169540, for 15 minutes prior to lysis and immunoprecipitation of ErbB1. Tyrosine phosphorylation of ErbB1 was assayed using an antiphosphotyrosine antibody (4G10; Upstate Biotechnology) and ErbB1 was quantified with an anti-Erb1 antibody (clone LA-22). (B) Myeloma cells were IL-6 starved for 3 hours and cultured in RPMI 1640 culture medium and 5% FCS with either 5 pg/mL IL-6 or 5 pg/mL IL-6 and 1 μg/mL HB-EGF, without or with 1 μM of the PD169540 ErbB inhibitor. Cells were cultured for 3 days and stained with FITC-annexin-V to determine the percentage of apoptotic cells. (C) Myeloma cell lines were IL-6 starved for 3 hours and cultured in RPMI 1640 culture medium and 5% FCS with 5 pg/mL IL-6 and 1 μg/mL HB-EGF without (□) or with (■) the PD169540 ErbB inhibitor (1 μM). Cells were cultured for 5 to 7 days and pulsed with tritiated thymidine at the end of the culture. Mean tritiated thymidine incorporations were determined in sixuplicate culture wells and results are expressed as percentages ± SDs of the mean proliferation without ErbB inhibitor. \*Indicates that the mean value is statistically significantly different from that obtained with 5 pg/mL IL-6 and HB-EGF without inhibitor, using a Student *t* test ( $P \leq .05$ ). (D) XG-1, XG-6, and XG-7 cells were IL-6 starved for 3 hours and cultured in RPMI 1640 culture medium and 5% FCS with a high concentration of IL-6 (500 pg/mL), without (□) or with (■) the PD169540 ErbB inhibitor (1 μM). Mean tritiated thymidine incorporations were determined in sixuplicate culture wells and results are expressed as percentages ± SDs of the mean proliferation without ErbB inhibitor. \*Indicates that the mean value is statistically significantly different from that obtained without inhibitor, using a Student *t* test ( $P \leq .05$ ). (E) XG-7 myeloma cells were starved overnight in culture medium without serum or cytokines with or without the PD169540 ErbB inhibitor. Cells were then stimulated with either no cytokine (Co), or IL-6 (20 ng/mL), or HB-EGF (5 μg/mL), or IGF-1 (1 μg/mL) for 15 minutes at 37°C. Lysates from unstimulated (Co) or stimulated myeloma cells were immunoblotted with anti-phospho-specific AKT antibody (top panel) and reprobed with anti-AKT antibody (bottom panel).

**Inhibition of HB-EGF-induced myeloma cell proliferation by ErbB inhibitors**

We then looked for the ability of ErbB inhibitors to block the HB-EGF-induced growth of myeloma cells. We used the PD169540 pan-ErbB kinase inhibitor, an analog of the CI-1033 clinical-grade inhibitor,<sup>25</sup> which at a 1-μM concentration is known to inhibit specifically ErbB1, ErbB2, and ErbB4 kinase activities, without affecting a large panel of other kinases (Dr Leopold, Pfizer Laboratories, personal communication, August 2002). Accordingly, PD169540 blocked ErbB1 phosphorylation induced by HB-EGF in A431 cells (Figure 6A). PD169540 reversed the HB-EGF-induced survival of myeloma cells (Figure 6B) and inhibited the HB-EGF-induced proliferation of the 6 myeloma cell lines that were sensitive to HB-EGF (Figure 6C). This inhibitor had no toxic effect on the growth induced by high IL-6 concentration of XG-6 cells, that failed to express the *HB-EGF* gene

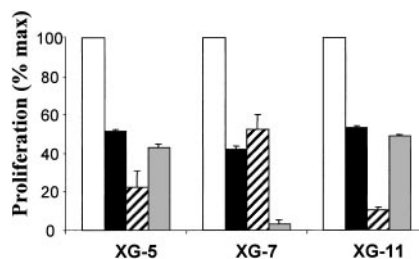
(Figure 6D). In the 2 cell lines expressing the *HB-EGF* gene, XG-1 and XG-7, PD169540 inhibitor partially blocked the proliferation induced by high IL-6 concentration, in agreement with our previous results obtained with anti-HB-EGF antibodies or CRM197 mutated diphtheria toxin (Figure 6D).<sup>4,5</sup> These data demonstrate again that acrotrine HB-EGF produced by some myeloma cell lines contributes to their growth induced by recombinant IL-6. As expected, the ErbB inhibitor blocked HB-EGF-induced AKT phosphorylation in XG-7 cells (Figure 6E). Its specificity is shown by its lack of effect on IGF-1-induced Akt phosphorylation (Figure 6E). Interestingly, it also inhibited IL-6-induced AKT phosphorylation. This observation is in agreement with the inhibition of IL-6-dependent proliferation of this HB-EGF-producing cell line by ErbB inhibitor (Figure 6D) and indicates a cooperation between gp130 and ErbB transduction pathways.

We then tested the other 2 inhibitors known to block ErbB1 tyrosine kinase activation, AG1478 and PD153035, on the 3 ErbB1-positive myeloma cell lines, XG-5, XG-7, and XG-11. These 2 inhibitors also blocked the HB-EGF-induced proliferation of the 3 ErbB1 myeloma cell lines in a manner similar to PD169540 (Figure 7). As all myeloma cell lines express the *ErbB2* gene and as ErbB2 can participate in the HB-EGF-induced signaling,<sup>36</sup> we also tested the Herceptin anti-Erb2 antibody.<sup>37</sup> We found that this antibody did not affect HB-EGF-induced myeloma cell proliferation (results not shown).

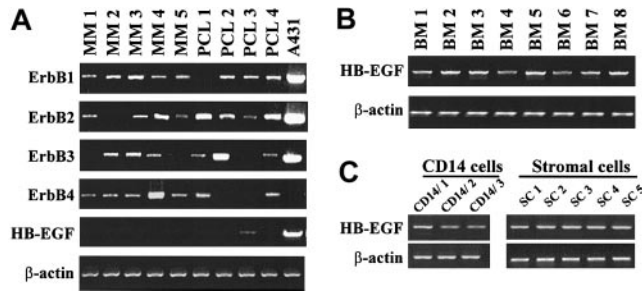
**The pan-ErbB kinase inhibitor potentiates dexamethasone-induced or anti-IL-6 MoAb-induced apoptosis of patient myeloma cells**

We first looked for the expression of ErbB receptors in primary purified myeloma cells from 5 patients with intramedullary myeloma and 4 patients with PCL. As indicated in Figure 8A, 7 of 9 purified primary myeloma cells expressed *ErbB4*. 8 of 9 expressed *ErbB1* and *ErbB2*, and 6 expressed *ErbB3*. Thus, 9 of 9 primary myeloma cells expressed either *ErbB1* and/or *ErbB4* and therefore might be sensitive to HB-EGF. Purified myeloma cells from 8 of 9 patients did not express the *HB-EGF* gene but the bone marrow mononuclear cells of 8 of 8 patients with MM expressed the *HB-EGF* gene (Figure 8B). Bone marrow CD14 monocytes were purified from 3 patients and bone marrow stromal cell lines were derived from 5 patients. It is noteworthy that *HB-EGF* expression was found in 3 of 3 purified bone marrow CD14 cells as well as in 5 of 5 stromal cell lines (Figure 8C).

We then investigated the effect of the pan-ErbB inhibitor on the survival of primary myeloma cells. Detailed results obtained with 8



**Figure 7. The myeloma cell growth factor activity of HB-EGF is inhibited by 2 ErbB1-specific inhibitors.** *ErbB1*-positive myeloma cell lines (XG-5, XG-7, and XG-11) were IL-6 starved for 3 hours and cultured in RPMI 1640 culture medium and 5% FCS with 5 pg/mL IL-6 and 1 μg/mL HB-EGF without (□) or with PD169540 (1 μM; ■), AG1478 (1 μM; ▨), and PD153035 (1 μM; ▩). Cells were cultured for 6 days and pulsed with tritiated thymidine at the end of the culture. Mean tritiated thymidine incorporations were determined in sixuplicate culture wells and results are expressed as percentages ± SDs of the mean proliferation without ErbB inhibitor. \*Indicates that the mean value is statistically significantly different from that obtained with 5 pg/mL IL-6 and HB-EGF without inhibitor, using a Student *t* test ( $P \leq .05$ ).



**Figure 8. Expression of the *ErbB* receptors and *HB-EGF* in purified primary myeloma cells and in the tumor environment.** (A) *ErbB1*, *ErbB2*, *ErbB3*, *ErbB4*, and *HB-EGF* expression was assayed by RT-PCR on primary myeloma cells. These cells were purified (> 95%) from 9 patients (5 with intramedullary myeloma and 4 with PCL). The A431 epidermoid carcinoma cell line was used as a control. (B) *HB-EGF* gene expression was analyzed by RT-PCR in unselected mononuclear cells from 8 patients with MM. (C) *HB-EGF* gene expression was analyzed by RT-PCR in CD14 purified monocytes (> 98%) from 3 patients and in stromal cells derived from 5 patients. Amplification of  $\beta$  actin shows the equivalence of the cDNA loading and amplification. Results are of one experiment representative of 3.

patients are shown in Table 1. In 6 of 8 patients, the B-E8 anti-IL-6 MoAb or the pan-ErbB kinase inhibitor significantly reduced the number of viable myeloma cells (respectively by 45% and 55%,  $P \leq .05$ ; Table 1 and Figure 9A). The effect of DEX was less marked and not significant ( $P = .07$ ). Of major interest, the pan-ErbB inhibitor dramatically increased both anti-IL-6 MoAb-induced or DEX-induced apoptosis. We observed a 76% reduction of the number of viable myeloma cells with anti-IL-6 MoAb and the ErbB inhibitor ( $P = .0028$ ) and a 84% reduction with DEX and the ErbB inhibitor ( $P = .0028$ ) (Figure 9A). In 2 patients, the 3 compounds had no effect on myeloma cell survival, either used alone or in combination (Table 1). Interestingly, the pan-ErbB

inhibitor had no effect on the survival of other bone marrow nonmyeloma cells (Figure 9B). We then looked for the effect of the pan-ErbB kinase inhibitor on the growth of hematopoietic precursors present in the bone marrow of 5 patients with MM using a methylcellulose semisolid assay (Figure 10A). None of the 3, pan-ErbB kinase inhibitor, DEX, or anti-IL-6 MoAb, significantly affected it, either used alone or in combination ( $P > .5$ ).

**Effect of *HB-EGF* inhibitor on adhesion of myeloma cells to BMSCs and IL-6 production**

As *HB-EGF* is expressed by MM patients' stromal cells and can be involved in juxtacrine intercellular communication,<sup>38</sup> we investigated whether the PD169540 could affect the adhesion of myeloma cell to BMSCs. About 20% myeloma cells adhered to BMSCs and pretreatment of both myeloma cells and BMSCs with the pan-ErbB inhibitor did not affect myeloma cell adhesion to stromal cells (Figure 10B). Given that binding of myeloma cells to BMSCs had been proven important to promote IL-6 production by stromal cells and myeloma cell survival,<sup>31,39</sup> we next investigated whether the PD169540 could affect the secretion of IL-6 by BMSCs cocultured with myeloma cells. IL-6 secretion was  $889 \text{ pg/mL} \pm 17 \text{ pg/mL}$  without ErbB inhibitor and  $810 \text{ pg/mL} \pm 43 \text{ pg/mL}$  with ErbB inhibitor. No IL-6 was detected in the supernatant of myeloma cells (results not shown). Therefore, the pan-ErbB inhibitor did not significantly affect the IL-6 production by stromal cells cultured with myeloma cells.

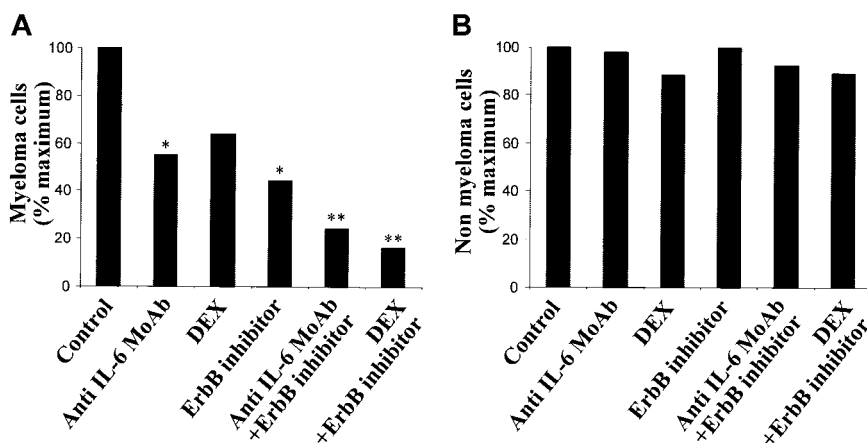
**Discussion**

As we initially found that the *HB-EGF* gene was expressed in some myeloma cell lines and could stimulate their growth,<sup>4,5</sup> our aim was

**Table 1. Effect of the pan-ErbB inhibitor on the survival of primary myeloma cells**

	Control	Anti-IL-6 MoAb	DEX	ErbB inhibitor	Anti-IL-6 MoAb + ErbB inhibitor	DEX + ErbB inhibitor
<b>Myeloma cell count/mL</b>						
Responder patients						
Patient 1	30 338	22 880	40 763	26 460	10 075	17 193
Patient 2	78 990	45 090	22 640	61 938	23 088	5 750
Patient 3	30 653	29 952	13 486	17 790	17 460	9 984
Patient 4	14 280	7 560	6 800	6 820	6 528	6 125
Patient 5	162 316	121 856	126 990	42 420	13 900	14 820
Patient 6	68 544	24 795	51 696	2 415	2 080	1 260
Median value	49 599	27 374	31 702	22 125	11 988	8 055
Nonresponder patients						
Patient 7	3 824	3 043	3 477	3 040	3 056	3 690
Patient 8	24 922	20 812	24 552	19 560	18 260	16 629
<b>Nonmyeloma cell count/mL</b>						
Responder patients						
Patient 1	344 663	302 120	334 238	323 540	314 925	307 808
Patient 2	221 010	254 910	257 360	248 062	216 912	244 250
Patient 3	259 347	230 048	206 514	282 210	282 540	230 016
Patient 4	325 720	292 440	293 200	303 180	233 472	243 875
Patient 5	177 684	218 144	213 010	167 580	186 100	185 180
Patient 6	271 456	265 205	188 304	227 585	257 920	198 740
Median value	265 402	260 058	235 185	265 136	245 696	236 946
Nonresponder patients						
Patient 7	156 176	166 957	186 523	196 960	156 944	146 310
Patient 8	315 078	199 188	285 448	220 440	201 740	213 371

Mononuclear cells from 8 patients with MM were cultured for 5 days in RPMI 1640 medium, 5% FCS, 1 ng/mL IL-6, with or without the PD169540 ErbB inhibitor (1  $\mu$ M), B-E8 anti-IL-6 MoAb (10  $\mu$ g/mL), and DEX (10<sup>-6</sup> M). At day 5 of culture, the viability and cell counts were assayed and the percentage of CD138<sup>+</sup> viable myeloma cells was determined by flow cytometry. Results are expressed as number of myeloma and nonmyeloma cells per milliliter. The median values were calculated from the 6 responder patients. Two patients did not significantly respond to the 3 compounds, either used alone or in combination



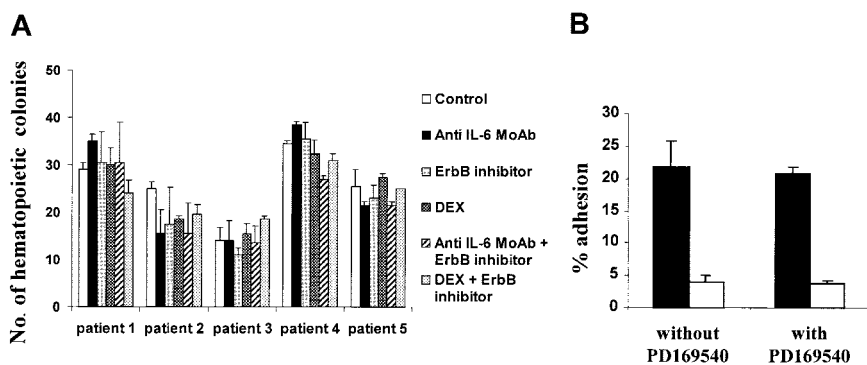
**Figure 9. The PD169540 ErbB inhibitor potentiates dexamethasone and anti-IL-6–induced apoptosis of primary myeloma cells.** Mononuclear cells from 8 patients with MM were cultured at  $5 \times 10^5$  cells/mL in RPMI 1640 medium and 5% FCS with either the PD169540 ErbB inhibitor ( $1 \mu\text{M}$ ) or anti-IL-6 MoAb (B-E8;  $10 \mu\text{g/mL}$ ) or dexamethasone (DEX,  $10^{-6} \text{M}$ ), alone or in combination, for 5 days. As endogenous production of IL-6 is highly variable in short-term culture of patients' bone marrow, 1 ng/mL recombinant IL-6 was added to eliminate this source of variability. In each culture group, viability and cell counts were assayed and myeloma cells were stained with an anti-CD138-PE antibody. Results are median values of the numbers of myeloma (A) or nonmyeloma cells (B), compared with that of the control group, which assigned the value of 100%. Only the 6 responder patients listed in Table 1 are represented there. \*Indicates a statistically significant difference of the median value compared with that of the control group using a Wilcoxon test for pairs ( $P \leq .05$ ). \*\*Indicates a statistically significant difference of the median value compared with that obtained with the anti-IL-6 MoAb or the DEX groups using a Wilcoxon test for pairs ( $P \leq .05$ ).

to investigate whether this observation was a common feature in multiple myeloma and whether ErbB inhibitors could be potentially useful in treating this disease.

First, we show here that *HB-EGF* expression is not a salient feature of primary myeloma cells. There is no *HB-EGF* gene expression in the purified myeloma cells of 8 of 9 patients with MM. *HB-EGF* expression was found in the myeloma cells of only one patient with PCL. An autocrine production of *HB-EGF* by myeloma cells could eventually make it possible to obtain a myeloma cell line in vitro, explaining why 2 myeloma cell lines of 9 express the *HB-EGF* gene. Interestingly, the bone marrow tumor environment of all 8 patients with MM expressed the *HB-EGF* gene. Monocytes were purified from 3 patients with MM and were found to express the *HB-EGF* gene. This was expected because stimulated monocytes are known to produce *HB-EGF*<sup>40</sup> and monocytes are highly activated in MM patients' bone marrow, in particular producing high levels of IL-6.<sup>41</sup> *HB-EGF* expression was also found in the 5 stromal cell lines derived from the bone marrow of patients with MM. We could not directly assay for a production of *HB-EGF* protein because a

sensitive *HB-EGF* ELISA is not commercially available. Thus, these current data emphasize that the bone marrow environment of patients with MM produces *HB-EGF*, in particular monocytes and stromal cells and that primary myeloma cells rarely express the *HB-EGF* gene.

Second, we found that myeloma cells from 6 of 9 myeloma cell lines and myeloma cells from 9 of 9 patients expressed the 2 *HB-EGF* receptor genes, *ErbB1* and/or *ErbB4*. Using RT-PCR, we did not detect ErbB expression in polyclonal plasmablasts generated from normal peripheral blood B cells using the methodology we recently reported.<sup>42</sup> In agreement with this study, we found that an ErbB inhibitor did not affect the generation of plasmablasts (results not shown). These results are intriguing and suggest that ErbB is either expressed on bone marrow plasmocytes, unlike plasmablasts, or involved in plasma cell oncogenesis. We are now trying to obtain purified bone marrow plasma cells to investigate this point, but these cells are rare cells (0.25% of bone marrow cells) and for ethical reasons, it is difficult to obtain enough normal bone marrow to purify them. The level of *ErbB* gene expression on myeloma cell lines



**Figure 10. The PD169540 ErbB inhibitor has no effect on the growth of the hematopoietic progenitors or on the adhesion of MM cells to stromal cells.** (A) Bone marrow mononuclear cells from 5 patients with MM were grown in a semisolid culture medium with hematopoietic cytokines for 14 days. Cells were treated with either the PD169540 pan-ErbB inhibitor ( $1 \mu\text{M}$ ) or anti-IL-6 MoAb (B-E8;  $10 \mu\text{g/mL}$ ) or  $10^{-6} \text{M}$  dexamethasone (DEX), alone or in combination. The number of granulocyte-macrophage colonies was counted on day 14 of incubation. Data are means  $\pm$  SD of the number of colonies determined on 2 different culture plates for each condition. (B) XG-11 cells were cultured with either BMSC-coated (■) or noncoated culture plates (□), without or with  $1 \mu\text{M}$  PD169540 for 24 hours. Nonadherent cells were counted and adherent cells were trypsinized and counted. In the 2 cell populations, GFP<sup>+</sup> stromal cells were quantified with FACS analysis. Results are expressed as percentage of bound plasma cells  $\pm$  SDs. The percentage was calculated as follows: percentage bound cells =  $([\text{input cells}] - [\text{nonadherent cells}] / [\text{input cells}] \times 100$ . Results are of one experiment representative of 3.

was low compared with A431 cells. In particular, ErbB1 protein was not detectable by immunoprecipitation in 2 myeloma cell lines, unlike in A431 cells. ErbB4 was detected in 2 of the 4 cell lines expressing the *ErbB4* gene. Despite this low ErbB expression, recombinant HB-EGF stimulated the growth of the 6 *ErbB1*<sup>+</sup> and/or *ErbB4*<sup>+</sup> cell lines, unlike the 3 *ErbB1*<sup>-</sup>/*ErbB4*<sup>-</sup> cell lines. We found that recombinant HB-EGF activity required a low concentration of IL-6 and was blocked by an antibody to IL-6. Recombinant HB-EGF alone stimulated the proliferation of XG-1 cells but this was due to their previously reported ability to produce a small amount of autocrine IL-6,<sup>33</sup> since HB-EGF-induced growth was blocked by an anti-IL-6 MoAb. These data are in agreement with recent data showing the ability of distinct cell surface receptors to cross-communicate. This cooperation between HB-EGF/ErbB and IL-6/gp130 pathways to trigger myeloma cell growth might be explained by the interaction of ErbB transducing elements with gp130, as it was reported previously in epithelial cancer cells. In a prostate carcinoma cell line, Qiu et al first demonstrated receptor crosstalk between the gp130 and ErbB3 via a gp130/ErbB2/ErbB3 complex.<sup>11</sup> Badache et al showed that IL-6-induced MAPK/PI3K activity depends on the cooperation with an EGFR autocrine loop in T47D breast cancer cells. In response to IL-6 treatment, the protein phosphatase Shp-2 and the docking molecule Gab1, which are constitutively associated with ErbB1, are recruited to gp130/JAK complexes and subsequently phosphorylated.<sup>12</sup> In addition, EGF was shown to enhance oncostatin M (OSM)-induced inhibition of cellular proliferation in the MCF-7 breast carcinoma cell line, where gp130 is constitutively associated with ErbB2 and ErbB3.<sup>43</sup> Recently, several examples of receptor crosstalk have been reported in multiple myeloma. IFN- $\alpha$  stimulation can induce tyrosine phosphorylation of gp130<sup>44</sup> or ErbB3<sup>15</sup> in a myeloma cell line. Podar et al showed that gp130 and IGF-1 receptor are colocalized in caveolae, which are cholesterol-rich plasma membrane microdomains.<sup>45</sup> These data suggest that these caveolin pits might play an important role in receptor interactions in multiple myeloma, as gp130 IL-6 transducer, IGF1-R, and eventually ErbB receptors and coreceptors might be colocalized in these membrane structures. Thus, the present study shows that HB-EGF is a third major myeloma cell growth factor, as are IL-6 and IGF-1.<sup>46</sup> Further studies are necessary to clarify how a low concentration of IL-6 and HB-EGF work together to promote myeloma cell growth.

The 2 known myeloma cell growth factors, IL-6 and IGF-1, activate 3 major transduction pathways in myeloma cells: the JAK/STAT pathway induced by IL-6, the MAPK, and the PI3-K/AKT pathway induced by both IL-6 and IGF-1.<sup>29,34,35,46,47</sup> An activation of these 3 pathways in our myeloma cells by IL-6 or IGF-1 was confirmed here. In agreement with the known activation of PI-3K/AKT by the ErbB receptor family in various cells,<sup>16</sup> HB-EGF induced AKT phosphorylation in myeloma cells. It did not activate STAT3 or MAPK phosphorylation. MAPK phosphorylation was looked for from 15 minutes to 24 hours after HB-EGF triggering, excluding a delayed activation. This lack of MAPK activation by HB-EGF was unexpected. Indeed, the ras/MAPK pathway is frequently activated by EGF ligands in various cells.<sup>16</sup> We will explore whether other signaling pathways known to be activated by HB-EGF in various cells (PCL $\gamma$ , PKC, Sarc kinase)<sup>48</sup> could be involved in HB-EGF-induced myeloma cell growth.

A major point was to determine whether inhibitors of ErbB kinases could promote apoptosis of primary myeloma cells and block the HB-EGF myeloma cell growth activity. As myeloma cells expressed ErbB4 and/or ErbB1, we used the PD169540 inhibitor, which inhibits kinase activity of ErbB1, ErbB2, and ErbB4, without affecting a large panel of other kinases. This inhibitor is close to the CI-1033 investigated in patients with neck, breast, and NSCL cancers.<sup>23</sup> This pan-ErbB kinase inhibitor blocked the HB-EGF-induced proliferation of the 6 sensitive cell lines at a 1- $\mu$ M concentration, which ensures specificity on ErbB members (Dr Leopold, Pfizer Laboratories, personal communication, August 2002). Two other ErbB1 inhibitors (AG1478 and PD153035) also blocked the HB-EGF-induced stimulation of the 3 ErbB1 myeloma cell lines. Despite *ErbB2* expression in myeloma cells, we found no inhibition of HB-EGF-induced myeloma cell proliferation by the Herceptin anti-ErbB2 MoAb. As ErbB2 participated in HB-EGF response in various cells,<sup>36</sup> this suggests that ErbB2 expression in myeloma cells is too weak to be functional. The PD169540 pan-ErbB inhibitor did not affect the IL-6-induced proliferation of the XG-6 myeloma cells that did not express the *HB-EGF* gene. It had no toxicity on the 14-day growth of bone marrow hematopoietic progenitors. As HB-EGF is a membrane protein that can be involved in juxtacrine stimulation and is produced by bone marrow stromal cells, we looked for a putative role of PD169540 on the interaction of myeloma cells and BMSCs in accordance with the model reported by Uchimaya et al.<sup>31</sup> We found that the ErbB inhibitor had no effect on myeloma cell adhesion to stromal cells or on IL-6 production by stromal cells cocultured with myeloma cells.

It is of major interest that the pan-ErbB kinase inhibitor induced a 55% reduction in the number of viable primary myeloma cells cultured for several days together with their bone marrow environment, which expresses the *HB-EGF* gene. In association with DEX, it induced an 84% reduction in myeloma cell number, whereas DEX alone induced only a 36% reduction. A similar additive effect was observed with an anti-IL-6 MoAb. Interestingly, this toxicity was specific to myeloma cells and no reduction in the number of nonmyeloma cells was observed. Several ErbB inhibitors are in various stages of clinical development and some are producing significant clinical responses. The compound that is at the most advanced stage of development is IRESSA (ZD1839), which targets EGFR. This compound has shown an acceptable tolerability and promising clinical efficacy in phase 1 trials and is currently in phase 3 trials in advanced NSCL and breast cancers.<sup>49</sup> CI-1033 is another clinical-grade irreversible tyrosine kinase inhibitor that is unique because it targets the 3 ErbB members with kinase activity.<sup>25</sup> It has potent and long-lasting *in vivo* activity in a range of tumor models. For example, it has demonstrated antitumor activity against A431 and H125 tumors *in vivo*.<sup>50</sup> Data from 3 phase 1 trials in patients with head and neck, breast, and NSCL cancers showed that CI-1033 is generally well tolerated.<sup>51</sup> A phase 2 study in patients with advanced ovarian cancer and a randomized phase 2 trial with patients with NSCL after a first or second line of chemotherapy are being done.<sup>52</sup>

In conclusion, our data are very encouraging and provide a framework for clinical evaluation of ErbB inhibitors in patients with MM, alone and coupled with dexamethasone or anti-IL-6 MoAb, now available for clinical use.<sup>53</sup> A first step will be to investigate in preclinical studies whether ErbB inhibitors can block the growth of myeloma cell lines and primary myeloma cells with *SCID* mice models of human multiple myeloma.<sup>27,54</sup> These current



data are in agreement with the recent development of novel biologically based treatment approaches that target mechanisms whereby MM cells grow and survive in their bone marrow environment. The most relevant example is the proteasome inhibitor PS341, which was identified as a promising new therapeutic agent in multiple myeloma.<sup>55</sup>

## Acknowledgment

We thank Dr Leopold (Pfizer Global Research and Development, Ann Arbor, MI) for providing us with the PD169540 pan-ErbB inhibitor.

## References

- Klein B, Zhang XG, Jourdan M, et al. Paracrine rather than autocrine regulation of myeloma-cell growth and differentiation by interleukin-6. *Blood*. 1989;73:517-526.
- Kawano M, Hirano T, Matsuda T, et al. Autocrine generation and essential requirement of BSF-2/IL-6 for human multiple myeloma. 1988;332:83-85.
- Gu ZJ, De Vos J, Rebouissou C, et al. Agonist anti-gp130 transducer monoclonal antibodies are human myeloma cell survival and growth factors. *Leukemia*. 2000;14:188-197.
- De Vos J, Couderc G, Tarte K, et al. Identifying intercellular signaling genes expressed in malignant plasma cells by using complementary DNA arrays. *Blood*. 2001;98:771-780.
- Wang YD, De Vos J, Jourdan M, et al. Cooperation between heparin-binding EGF-like growth factor and interleukin-6 in promoting the growth of human myeloma cells. *Oncogene*. 2002;21:2584-2592.
- Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J*. 1997;16:1647-1655.
- Harris RC, Chung E, Coffey RJ. EGF receptor ligands. *Exp Cell Res*. 2003;284:2-13.
- Falls DL. Neuregulins: functions, forms, and signaling strategies. *Exp Cell Res*. 2003;284:14-30.
- Yamauchi T, Ueki K, Tobe K, et al. Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. *Nature*. 1997;390:91-96.
- Prenzel N, Zwick E, Daub H, et al. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*. 1999;402:884-888.
- Qiu Y, Ravi L, Kung HJ. Requirement of ErbB2 for signalling by interleukin-6 in prostate carcinoma cells. *Nature*. 1998;393:83-85.
- Badache A, Hynes NE. Interleukin 6 inhibits proliferation and, in cooperation with an epidermal growth factor receptor autocrine loop, increases migration of T47D breast cancer cells. *Cancer Res*. 2001;61:383-391.
- Roudabush F, Pierce K, Maudsley S, Khan K, Luttrell L. Transactivation of the EGF receptor mediates IGF-1-stimulated shc phosphorylation and ERK1/2 activation in COS-7 cells. *J Biol Chem*. 2000;275:22583-22589.
- Hurbin A, Dubrez L, Coll J, Favrot M. Inhibition of apoptosis by amphiregulin via an insulin-like growth factor-1 receptor-dependent pathway in non-small cell lung cancer cell lines. *J Biol Chem*. 2002;277:49127-49133.
- Walters D, French J, Arendt B, Jelinek D. A typical expression of ErbB3 in myeloma cells: cross-talk between ErbB3 and the interferon- $\alpha$  signaling complex. *Oncogene*. 2003;22:3598-3607.
- Olayioye MA, Neve RM, Lane HA, Hynes NE. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J*. 2000;19:3159-3167.
- Yarden Y. The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities. *Eur J Cancer*. 2001;37(suppl 4):S3-S8.
- Holbro T, Civenni G, Hynes NE. The ErbB receptors and their role in cancer progression. *Exp Cell Res*. 2003;284:99-110.
- Nicholson R, Gee J, Harper M. EGFR and cancer prognosis. *Eur J Cancer*. 2001;37 (suppl 4):S9-S15.
- Carpenter G. ErbB-4: mechanism of action and biology. *Exp Cell Res*. 2003;284:66-77.
- Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*. 1989;244:707-712.
- Bodey B, Bodey B Jr, Groger AM, et al. Clinical and prognostic significance of the expression of the c-erbB-2 and c-erbB-3 oncoproteins in primary and metastatic malignant melanomas and breast carcinomas. *Anticancer Res*. 1997;17:1319-1330.
- Fry DW. Mechanism of action of erbB tyrosine kinase inhibitors. *Exp Cell Res*. 2003;284:131-139.
- Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene*. 2000;19:6550-6565.
- Slichenmyer WJ, Elliott VL, Fry DW. CI-1033, a pan-erbB tyrosine kinase inhibitor. *Semin Oncol*. 2001;28:80-85.
- Zhang XG, Gaillard JP, Robillard N, et al. Reproducible obtaining of human myeloma cell lines as a model for tumor stem cell study in human multiple myeloma. *Blood*. 1994;83:3654-3663.
- Rebouissou C, Wijdenes J, Autissier P, et al. A gp130 interleukin-6 transducer-dependent SCID model of human multiple myeloma. *Blood*. 1998;91:4727-4737.
- Jourdan M, De Vos J, Mechti N, Klein B. Regulation of Bcl-2-family proteins in myeloma cells by three myeloma survival factors: interleukin-6, interferon-alpha and insulin-like growth factor 1. *Cell Death Differ*. 2000;7:1244-1252.
- De Vos J, Jourdan M, Tarte K, Jasmin C, Klein B. JAK2 tyrosine kinase inhibitor tyrphostin AG490 down-regulates the MAPK and STAT pathways and induces apoptosis in myeloma cells. *Br J Haematol*. 2000;109:823-828.
- Sun RX, Lu ZY, Wijdenes J, et al. Large scale and clinical grade purification of syndecan-1<sup>+</sup> malignant plasma cells. *J Immunol Methods*. 1997;205:73-79.
- Uchiyama H, Barut BA, Mohrbacher AF, Chauhan D, Anderson KC. Adhesion of human myeloma-derived cell lines to bone marrow stromal cells stimulates interleukin-6 secretion. *Blood*. 1993;82:3712-3720.
- Stoll SW, Kansra S, Peshick S, et al. Differential utilization and localization of ErbB receptor tyrosine kinases in skin compared to normal and malignant keratinocytes. *Neoplasia*. 2001;3:339-350.
- Jourdan M, Zhang XG, Portier M, et al. IFN-alpha induces autocrine production of IL-6 in myeloma cell lines. 1991;147:4402-4407.
- Shi Y, Hsu JH, Hu L, Gera J, Lichtenstein A. Signal pathways involved in activation of p70S6K and phosphorylation of 4E-BP1 following exposure of multiple myeloma tumor cells to interleukin-6. *J Biol Chem*. 2002;277:15712-15720.
- Hideshima T, Nakamura N, Chauhan D, Anderson KC. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. *Oncogene*. 2001;20:5991-6000.
- Iwamoto R, Yamazaki S, Asakura M, et al. Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc Natl Acad Sci U S A*. 2003;100:3221-3226.
- Leyland-Jones B. Trastuzumab: hopes and realities. *Lancet Oncol*. 2002;3:137-144.
- Iwamoto R, Mekada E. Heparin-binding EGF-like growth factor: a juxtacrine growth factor. *Cytokine Growth Factor Rev*. 2000;11:335-344.
- Lokhorst HM, Lamme T, de Smet M, et al. Primary tumor cells of myeloma patients induce interleukin-6 secretion in long-term bone marrow cultures. *Blood*. 1994;84:2269-2277.
- Nakano T, Raines EW, Abraham JA, Klagsbrun M, Ross R. Lysophosphatidylcholine upregulates the level of heparin-binding epidermal growth factor-like growth factor mRNA in human monocytes. *Proc Natl Acad Sci U S A*. 1994;91:1069-1073.
- Portier M, Rajzbaum G, Zhang XG, et al. In vivo interleukin-6 gene expression in the tumoral environment in multiple myeloma. *Eur J Immunol*. 1991;21:1759-1762.
- Tarte K, De Vos J, Thykjaer T, et al. Generation of polyclonal plasmablasts from peripheral blood B cells: a normal counterpart of malignant plasmablasts. *Blood*. 2002;100:1113-1122.
- Grant SL, Hammacher A, Douglas AM, et al. An unexpected biochemical and functional interaction between gp130 and the EGF receptor family in breast cancer cells. *Oncogene*. 2002;21:460-474.
- French J, Walters D, Jelinek D. Transactivation of gp130 in myeloma cells. *J Immunol*. 2003;170:3717-3723.
- Podar K, Tai Y, Cole C, et al. Essential role of caveolae in interleukin-6- and insulin-like growth factor I-triggered Akt-1-mediated survival of multiple myeloma cells. *J Biol Chem*. 2003;278:5794-5801.
- Ferlin M, Noraz N, Hertogh C, et al. Insulin-like growth factor induces the survival and proliferation of myeloma cells through an IL-6-independent transduction pathway. *Br J Haematol*. 2000;111:626-634.
- Qiang YW, Kopantzev E, Rudikoff S. Insulinlike growth factor-I signaling in multiple myeloma: downstream elements, functional correlates, and pathway cross-talk. *Blood*. 2002;99:4138-4146.
- Jorissen RN, Walker F, Pouliot N, et al. Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res*. 2003;284:31-53.
- Ranson M. ZD1839 (Iressa) for more than just non-small cell lung cancer. *Oncologist*. 2002;7:16-24.
- Driscoll D, Steinkampf R, Patmore S, Elliott W, Klohs W. Effect of epidermal growth factor receptor tyrosine kinase inhibitor PD183805 on vascular endothelial growth factor secretion from several tumor models. *Proc Am Assoc Cancer Res*. 1999;40:121.
- Shin DM, Nemanitis J, Zinner RG, et al. A phase I clinical and biomarker study of CI-1033, a novel pan-ErbB tyrosine kinase inhibitor in patients with solid tumors. *Proc Am Soc Clin Oncol*. 2001;21:82.
- Sridhar S, Seymour L, Shepherd F. Inhibitors of epidermal-growth-factor receptors: a review of clinical research with a focus on non-small-cell lung cancer. *Lancet Oncol*. 2003;4:397-406.
- Trikha M, Corringham R, Klein B, Rossi J. Targeted anti-IL-6 monoclonal antibody therapy for cancer: a review of the rationale and clinical evidence. *Clinical Cancer Research*. 2003;9:4653-4665.
- Yaccoby S, Barlogie B, Epstein J. Primary myeloma cells growing in SCID-hu mice: a model for studying the biology and treatment of myeloma and its manifestations. *Blood*. 1998;92:2908-2913.
- Richardson PG, Barlogie B, Berenson J, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. *N Engl J Med*. 2003;348:2609-2617.