The Outcome of Heregulin-induced Activation of Ovarian Cancer Cells Depends on the Relative Levels of HER-2 and HER-3 Expression

Fengji Xu, Yinhua Yu, Xiao-Feng Le, Cinda Boyer, Gordon B. Mills, and Robert C. Bast, Jr.2
Division of Medicine, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [F. X., Y. Y., X-F. L., G. B. M., R. C. B.], and Duke University Medical Center, Durham, North Carolina 27710 [C. B.]

ABSTRACT
Members of the epidermal growth factor receptor family of tyrosine kinases, including epidermal growth factor receptor, c-erbB-2 (HER-2), c-erbB-3 (HER-3), and c-erbB-4 (HER-4), can be coexpressed at different levels in nonhematopoietic tissues. Amplification and overexpression of HER-2 is found in approximately one-third of cancers that arise in the breast and ovary. In our previous studies, heregulin (HRG) and anti-HER-2 antibodies inhibited proliferation, increased invasiveness, and enhanced tyrosine autophosphorylation of SKBr3 breast cancer cells that overexpressed HER-2. In the present report, the effects of HRG and anti-HER-2 antibody have been compared in six ovarian cancer cell lines. HRG inhibited anchorage-independent growth of SKOv3 cells that overexpressed HER-2 (105 receptors/cell) but stimulated the growth of OVCA420, OVCA429, OVCA432, OVCA433, and OVCAR-3 cells that expressed lower levels of the receptor (104 receptors/cell) but stimulated the growth of OVCA420, OVCA429, OVCA432, OVCA433, and OVCAR-3 cells that expressed lower levels of the receptor (104 receptors/cell). Thus, cell lines with a high level of HER-2 relative to HER-3 appear to determine the response to HRG. Taken together, these studies support the concept that the level of HER-2 expression can modulate response to HRG, determining whether the response is stimulatory or inhibitory. In contrast, agonistic antibodies that bind to HER-2 alone inhibit anchorage-independent growth but fail to mimic HRG’s ability to stimulate growth of cells with low HER-2: HER-3 ratios.

INTRODUCTION
Four members of the EGFR1 family of tyrosine kinase growth factor receptors have been identified: (a) EGFR; (b) c-erbB-2 (HER-2); (c) c-erbB-3 (HER-3); and (d) c-erbB-4 (HER-4). These four receptor proteins are normally coexpressed at different levels in diverse tissues, excluding the hematopoietic system. Aberrant expression of EGFR has been observed in a variety of human tumors. In breast and ovarian cancer, increased or persistent expression of the EGFR has been associated with a poor prognosis (1, 2). HER-2 gene amplification and overexpression is found in approximately one-third of breast and ovarian cancers. Overexpression of HER-2 has been associated with a poor prognosis in node-positive breast cancers and in many, but not all, studies of advanced ovarian cancer (3, 4). The prognostic significance of levels of HER-3 and HER-4 expression is less well defined.

HRG was initially isolated during the search for a ligand that binds to the HER-2 receptor. The several isoforms of HRG bind to cells that express HER-3 or HER-4 alone, but not to cells that express only HER-2 (5–7). However, HRG can bind to heterodimers of HER-2 and HER-3 or HER-2 and HER-4 (8–10). Interactions between HER-2 and HER-3 may be important for HRG-induced signaling in that HER-3 lacks tyrosine kinase activity but contains multiple SH2 binding sites. HER-2 and HER-3 stimulated rather than inhibited growth. To evaluate the relative importance of HER-3 and HER-4, NIH 3T3 cells were cotransfected with HER-2 and HER-3 or with HER-2 and HER-4. HRG inhibited the growth of cells with a high ratio of HER-2:HER-3, whereas HRG stimulated the growth of cells with low levels of the two receptors. In cells that express only HER-2 and HER-4, HRG stimulated the growth of cells that expressed HER-4 independent of HER-2 levels. Anti-HER-2 antibodies inhibited the growth of transfectants with high levels of HER-2 expression independent of HER-3 or HER-4 expression. In ovarian cancer cells that express all three receptors, the relative levels of HER-2 and HER-3 appear to determine the response to HRG. Taken together, these studies support the concept that the level of HER-2 expression can modulate response to HRG, determining whether the response is stimulatory or inhibitory. In contrast, agonistic antibodies that bind to HER-2 alone inhibit anchorage-independent growth but fail to mimic HRG’s ability to stimulate growth of cells with low HER-2: HER-3 ratios.

Received 1/28/99; revised 8/10/99; accepted 8/17/99.
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.

1 Supported by Grant RO1 CA 39930 from the NIH, Department of Health and Human Services.
2 To whom requests for reprints should be addressed, at Division of Medicine, University of Texas M. D. Anderson Cancer Center, Box 092, 1515 Holcombe Boulevard, Houston, TX 77030.

3 The abbreviations used are: EGFR, epidermal growth factor receptor; HRG, heregulin; FBS, fetal bovine serum; TCM, tissue culture medium.
HER-4 are capable of phosphorylating multiple substrates but contain a limited repertoire of SH2 binding sites.

Our previous studies have demonstrated that HRG and anti-HER-2 antibodies inhibit proliferation, increase invasiveness, and enhance tyrosine autophosphorylation of breast cancer cells that overexpress HER-2, such as SKBr3 cells (11, 12). Other investigators have reported that HRG stimulates the growth of breast cancer cells that express low levels of the HER-2 receptor (13), but that ovarian cancer cells may be refractory to growth stimulation by the ligand (14). The present study documents that HRG can, in fact, stimulate anchorage-independent growth of ovarian cancer cells. The ratio of HER-2:HER-3 appears to be important in determining whether cells that express both receptors are stimulated or inhibited by HRG. In cells that express only HER-2 and HER-4, HRG stimulates cell proliferation independent of HER-2 levels. When all three receptors are present, the ratio of HER-2:HER-3 appears to be a critical determinant in regulating clonogenic growth.

MATERIALS AND METHODS

Cell Lines, Antibodies, and HRG. Human epithelial ovarian cancer cell lines OVCA420, OVCA429, OVCA432, and OVCA433 were maintained in MEM supplemented with 10% FBS, 2 mm L-glutamine, nonessential amino acids, 1 mm sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin (TCM). OVCAR-3 was maintained in RPMI 1640 supplemented with 10% FBS, 2 mm L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. SKOv3 was maintained in McCoy 5A medium containing 10% FBS, 2 mm L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The NIH 3T3 murine fibroblast cell line and the 17313 cell line (15) were kindly provided by Dr. B. D. Cohen (Bristol-Myers Squibb, Seattle, WA). Both cell lines were cultured in DMEM supplemented with 10% FBS and 2.5 mM histidinol (Sigma, St. Louis, MO). For all experiments, cells were detached with 0.25% trypsin-0.02% EDTA and washed once in complete medium before use.

Murine monoclonal antibodies to the extracellular domain of p185 were obtained from Applied BioTechnology/Oncogene Science (TA1, ID5, RC1, RC6, NB3, BD5, PB3, and OD3) and from Chiron, Inc. (Emeryville, CA; 454C11, 741F8, and 736G9). All antibodies were of the IgG1 isotype except PB3 (IgG2a) and OD3 (IgM). MOPC21 (IgG1) was used as an isotype-matched control that did not bind to p185. The H3.105 anti-HER-3 antibody was purchased from NeoMarker (Fremont, CA) and the 10-4 and 6-4-11 anti-HER-4 antibodies were generously provided by Dr. B. D. Cohen. Antibodies against HRG (including α and β types) and phosphotyrosine were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively.

Recombinant HRG β was obtained from Genentech (South San Francisco, CA).

Antibody and HRG Binding to EGFR and HER-2, HER-3, and HER-4 Receptors. A live cell radioimmunoassay was used to determine the binding of monoclonal antibodies and HRG to different cell lines and transfectants. Cells were trypsinized and seeded at a density of 2 × 10^4 cells/well in 96-well removable plates. After overnight incubation, monolayers were washed with 1% FBS in TCM supplemented with 0.1% sodium azide. For indirect binding assays, different monoclonal antibodies (10 μg/ml) were added in volumes of 50 μl to cell monolayers. After incubation at 4°C for 2 h, 125I-labeled sheep antirabbit αF(ab')2 fragment (200,000 cpm/well) was added in a volume of 100 μl and incubation was continued for another 2 h. Nonspecific binding was determined by adding...
125I-labeled sheep antimouse antibody F(ab’)2 fragment (50 µl) to cells without monoclonal antibodies or to empty wells. After incubation on ice for 4 h, unbound antibodies were removed by washing the wells four times with ice-cold TCM containing 5% FBS with 0.1% sodium azide. Individual wells were then detached, and radioactivity was determined in a Packard gamma counter. For direct binding assays, TA1, H3-105, 10-4 monoclonal antibodies, and HRG were directly labeled with125I using the Iodogen technique and incubated with different cell lines. The EBDA program was used to calculate the number of binding sites/cell (16, 17).

Transfection of HER-2, HER-3, and HER-4 cDNA. Plasmid 9002, containing the full-length human HER-2 gene, was obtained from Applied BioTechnology/Oncogene Science, Inc. Plasmid CHER-3x, containing the full-length human HER-3 gene, was a gift from Dr. Greg Plowman (SUGEN, Inc., Redwood City, CA). Plasmid H4y, containing the full-length human HER-4 gene, was provided by Dr. B. D. Cohen. NIH 3T3 cells and OVCAR-3 cells were transfected by Lipofectamin as directed by the manufacturer (Life Technologies, Inc.). At least seven independent transfectants were cloned and cultured continuously with G418 or hygromycin. Transfected cells were selected for their ability to bind antibodies directed against HER-2, HER-3, or HER-4.

Assays of Anchorage-independent Growth. Anchorage-independent cell growth was measured in 35-mm tissue culture dishes (Nunc, Inc., Naperville, IL). A 1-ml layer of 0.6% agar (Difco, Detroit, MI) in TCM was solidified in the bottom of
RESULTS

Expression of EGFR, HER-2, HER-3, and HER-4 in Ovarian Cancer Cell Lines. Six ovarian cancer cell lines were characterized for expression of EGFR, HER-2, HER-3, and HER-4. The 225, TAI, H3-105, and 10-4 antibodies that recognized each of these receptors were directly labeled with \(^{125}\text{I}\) and incubated with each cell line, and the number of binding sites was estimated (Fig. 1). The number of binding sites/cell was taken as an estimate of the receptor level. All six cell lines express intermediate levels of EGFR (10\(^{5}\)–10\(^{6}\) receptors/cell) and low levels of HER-3 and HER-4 (10\(^{3}\)–10\(^{4}\) receptors/cell). One of the six cell lines (OVCA-3) had low levels of HER-2, with <10\(^{3}\) receptors/cell; four of the six cell lines (OVCA420, OVCA429, OVCA432, and OVCA433) had intermediate expression of HER-2, with 10\(^{5}\)–10\(^{6}\) receptors/cell; whereas SKOv3 had 10–100-fold higher levels of HER-2, with 10\(^{7}\)–10\(^{8}\) receptors/cell (Fig. 1).

Binding of HRG to Ovarian Cancer Cells. Using a direct radioimmunoassay, we estimated the number of HRG binding sites/cell associated with different ovarian cancer cell lines (Fig. 1). Similar binding of HRG was observed in each of the six cell lines, although p185\(^{\text{HER-2}}\) levels varied from 10\(^{3}\)–10\(^{6}\) receptors/cell, consistent with the possibility that HER-2 did not contribute substantially to HRG binding.

Reactivity of Monoclonal Antibodies with EGFR and HER-2, HER-3, and HER-4 Receptors. Our previous studies defined the reactivity of 11 monoclonal antibodies with the extracellular domain of p185\(^{\text{HER-2}}\) and measured their ability to inhibit the growth of SKBr3 breast cancer cells (11). To further investigate all four EGFR family members, these 11 monoclonal antibodies and one additional anti-HER-2 antibody, 736G9, were incubated with NIH 3T3 cell lines that had been transfected individually with EGFR, HER-2, HER-3, or HER-4. Binding of antibodies was measured indirectly with \(^{125}\text{I}\)-labeled sheep antimouse F(ab\(^{\prime}\))\(_{2}\). Each of these 12 monoclonal antibodies bound strongly to the HER-2 receptor, but not to the EGF, HER-3, or HER-4 receptors. The 225 anti-EGFR, H3-105 anti-HER-3, and 10-4 anti-HER-4 antibodies bound strongly to the relevant receptors, but not to other receptors in this family. HRG bound to cells that expressed HER-3 or HER-4, but not to cells that expressed EGFR or HER-2 alone, consistent with previous reports (18).

Stimulation or Inhibition and Anchorage-independent Growth with HRG. The effect of HRG on anchorage-independent growth was measured in each of the six ovarian cancer cell lines. HRG stimulated growth in five of the six cell lines but inhibited the growth of SKOv3 cells that overexpressed HER-2
Stimulation or inhibition was dependent on the concentration of HRG. As much as 10 ng/ml HRG was required to produce significant growth inhibition in SKOv3 cells. To evaluate the possible contribution of endogenous HRG expression to growth regulation, we have measured relative HRG expression in six ovarian cancer cell lines by Western blotting (Fig. 4). Each of the six cell lines expressed the 44-kDa HRG protein. OVCAR-3 cells exhibited the highest level of HRG expression, and OVCA432 had the lowest level among these six cell lines. Consequently, no correlation was found between the response to HRG treatment and the endogenous expression of HRG protein.

Interaction with ligand and with antibody has been shown to activate the HER-2 kinase (11). HER-4 can also be activated by ligand, whereas HER-3 lacks kinase activity but can be phosphorylated by interaction with other members of the HER family (18). To explore the possibility that differences in phosphorylation of receptors might correlate with growth stimulation or inhibition, the phosphorylation status of HER-2, HER-3, and HER-4 was measured after treatment with HRG. As shown in Fig. 5, increased phosphorylation of HER-2 after HRG treatment was observed in every cell line. Increased phosphorylation of HER-3 after HRG treatment was observed in OVCAR-3, OVCA420, OVCA429, and OVCA432, whereas little, if any, phosphorylation of HER-3 was observed in SKOv3 and OVCA433. Increased phosphorylation of HER-4 after HRG treatment was observed only in OVCAR-3 cells (Fig. 5). Thus, no clear correlation was observed between the impact of HRG on clonogenic growth and tyrosine phosphorylation of HER-2, HER-3, and HER-4.

The monoclonal antibody ID5, which binds to the extracellular domain of p185 HER-2, inhibits the anchorage-independent growth of cells that overexpress HER-2 (19). Incubation with ID5 failed to inhibit or to stimulate growth in the five ovarian cancer cell lines that exhibited low levels of p185HER-2 but did inhibit the anchorage-independent growth of SKOv3 cells that expressed $10^5$–$10^6$ receptors/cells (Fig. 2). Incubation with HRG and ID5 did not prevent HRG-induced stimulation of anchorage-independent growth of the five cell lines with low levels of HER-2 expression (Figs. 2 and 3), and the ID5 antibody did not block HRG binding (data not shown).

**Effects of HRG on Transfected Ovarian Cancer Cells with Different Levels of HER-2 Expression.** Because the ovarian cancer cell lines were derived from different patients, an apparent correlation between p185HER-2 expression and the effect of HRG on anchorage-independent growth might relate to other abnormalities in the cells. Consequently, we have examined ovarian cancer cells and transfectants in which HER-2 expression could be up-regulated or down-regulated on the same background. OVCAR-3 cells express $10^3$ HER-2 receptors/cell and are stimulated by HRG. Transfection of OVCAR-3 cells with full-length HER-2 cDNA produced the OVU15 clone that expressed $10^5$ p185HER-2 sites/cell. Expression of HER-3 and HER-4 was similar in the transfectants and in parental cells. HRG stimulated the growth of the parental OVCAR-3 cells...
2–4-fold but inhibited the growth of the OVU15 cells with greater p185HER-2 expression (Fig. 6).

Introduction of E1A into cells that overexpress HER-2 can lower HER-2 expression and inhibit tumor cell growth (20). The SKOv3 cell line that overexpressed HER-2 was transfected with E1A to produce E1A-SKOv3 cells that had substantially lower levels of p185HER-2 (Fig. 7). Similar levels of HER-3 and HER-4 were found once again in the parental and transfected cells. HRG at high concentrations inhibited the growth of parental SKOv3 cells but stimulated the anchorage-independent growth of transfectants with decreased HER-2 expression.

**Effect of HRG on NIH 3T3 Cells Transfected with HER-2, HER-3, and/or HER-4.** Ovarian carcinoma cell lines express different levels of EGFR, HER-2, HER-3, and HER-4. HRG can interact with multiple receptors and permit crosstalk to occur (9, 21, 22). Consequently, we have studied interactions of HRG with NIH 3T3 cells that have been transfected with HER-2, HER-3, or HER-4 cells, alone or in combination. HRG failed to stimulate NIH 3T3 cells that had been transfected with HER-2 or HER-3 alone (data not shown). By contrast, a 5–7-fold stimulation of colony formation in anchorage-independent assays was observed when cells transfected with HER-4 were treated with HRG (Fig. 8).

In transfectants that expressed HER-2 and HER-3, the ratio of the two receptors proved to be important. With high levels of HER-2 (≥10⁵) and low levels of HER-3 (clone E21), HRG and ID5 inhibited anchorage-independent growth. When similar levels of HER-2 and HER-3 were expressed (clone E22), HRG, but not ID5, stimulated cell growth (Fig. 9). Transfectants with intermediate levels of HER-2 (clone E6) exhibited a lower level of growth stimulation by HRG (Fig. 9). Consequently, the inhibition or stimulation of anchorage-independent growth by HRG appeared to depend on the ratio of HER-2:HER-3 expression in cells that bore only these two receptors. Transfectants...
that contained combinations of HER-2 and HER-4 were stimulated regardless of the ratio of HER-2:HER-4 (Fig. 8). The anti-HER-2 ID5 antibody regularly inhibited clonogenic growth in cells that expressed 10^6 HER-2 receptors/cell.

**DISCUSSION**

In our earlier studies, HRG and certain anti-HER-2 antibodies inhibited clonogenic growth of the SKBr3 breast cancer cell line that overexpressed HER-2 (19). In the present study, clonogenic growth of SKOv3, an ovarian cancer cell line that overexpressed HER-2, was similarly inhibited by HRG and an anti-HER-2 antibody. Conversely, HRG stimulated anchorage-independent growth of five ovarian cancer cell lines that expressed lower levels of HER-2. All six cell lines expressed modest but demonstrable levels of HER-3 and HER-4. All six cell lines bound HRG and exhibited similar densities of binding sites for this ligand, consistent with the previously reported importance of HER-3 and HER-4 for binding HRG (18). In contrast to a previous report (14), it appears that HRG is capable of regulating the growth of ovarian cancer cell lines. The apparent difference between reports may relate to the different assays used to monitor growth of cancer cells.

In the clinic, the adverse prognostic significance of HER-2 overexpression appears greatest in node-positive breast cancer (1, 3) and in late-stage ovarian cancer (4). Although it may seem paradoxical that clonogenic growth can be inhibited by ligand and by agonistic anti-HER antibodies in cells that overexpress the receptor, recent data suggest that agonists increase the invasiveness of cells that overexpress HER-2 (12). Consequently, enhanced potential for invasion and metastasis, rather than an increased rate of growth or clonogenicity, may be associated with HER-2 overexpression. However, HRG might exert autocrine growth stimulation in cells with lower levels of HER-2, and the prognostic significance of HRG expression in cells with normal HER-2 levels deserves further study.

Overexpression of HER-2 has been found in approximately 30% of ovarian cancer (4), whereas overexpression of HER-3 and HER-4 has not been reported to date. Consistent with these studies of cancer tissues, HER-2 levels in the six ovarian cancer cell lines varied over 3 orders of magnitude, whereas the levels of EGFR, HER-3, and HER-4 varied by only 1 order of magnitude. Thus, marked heterogeneity of expression was observed with only one of the four members of the HER family of receptors. A corollary of this observation is that different levels of HER-2 expression will produce markedly different ratios of HER-2:HER-3 and HER-2:HER-4. When HRG was incubated with each of the six cell lines, growth inhibition appeared to correlate with high levels of HER-2 that produced high HER-2:HER-3 and HER-2:HER-4 ratios. Inhibition or stimulation of growth with HRG did not correlate with endogenous HRG expression or with HRG-induced phosphorylation of HER-2, HER-3, or HER-4. Consequently, downstream effects of HER-2 overexpression must contribute to the growth inhibition observed.

The effect of HRG in NIH 3T3 cells transfected with HER-2 and HER-3 or HER-2 and HER-4 suggest that the ratio of HER-2:HER-3 is important for cell growth regulation, whereas the ratio of HER-2:HER-4 is not. In OVCAR-3 cells and SKOV-3 cells that express all three receptors, modulation of HER-2 levels altered the response to HRG and to agonistic anti-HER-2 antibody. Our data suggest that the interaction of HER-2 and HER-3 may be particularly important for HRG or antibody-induced growth inhibition, overriding the stimulation produced by HER-4 homodimers or HER-2/HER-4 heterodimers. However, our data do not permit us to distinguish between the importance of the ratio of HER-2:HER-3 and the absolute level of HER-2 in determining response to HRG.

Anti-HER-2 antibodies inhibited the growth of cells with high levels of HER-2, regardless of HER-3 or HER-4 expression. Antibody-mediated growth stimulation was not observed.
The ID5 antibody binds to HER-2 alone and presumably signals predominantly through this receptor. HRG can signal through HER-4 homodimers, through HER-2/HER-3 heterodimers, or through HER-2/HER-4 heterodimers. Our results pose the interesting possibility that signaling through HER-2 alone inhibits clonogenic growth, and that this is recognized most readily in cells that overexpress HER-2. Additional studies are underway to delineate signaling pathways in cells treated with HRG or with agonistic HER-2 antibodies. Both agents can induce autophosphorylation of HER-2 and activate mitogen-activated protein kinase. The ID5 anti-HER-2 antibody, but not HRG, increases activity of phospholipase-Cγ and perturbs diacylglycerol levels. HRG, but not ID5, increases phosphorylation of HER-3/erbB3 and c-jun-NH2-kinase activity, as well as inducing differentiation. Thus, HRG and ID5 signal by different pathways.

REFERENCES


Xiao-Feng Le et al., unpublished data.
The Outcome of Heregulin-induced Activation of Ovarian Cancer Cells Depends on the Relative Levels of HER-2 and HER-3 Expression

Fengji Xu, Yinhua Yu, Xiao-Feng Le, et al.


Updated version Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/5/11/3653

Cited articles This article cites 20 articles, 7 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/5/11/3653.full.html#ref-list-1

Citing articles This article has been cited by 15 HighWire-hosted articles. Access the articles at:
/content/5/11/3653.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.