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Biologic effects of heregulin/*neu* differentiation factor on normal and malignant human breast and ovarian epithelial cells

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The heregulins are a family of ligands with ability to induce phosphorylation of the p185[HER-2/neu] receptor. Various investigators have reported a variety of responses of mouse and human breast and ovarian cells to this family of ligands including growth stimulation, growth inhibition, apoptosis and induction of differentiation in cells expressing the HER-2/neu receptor. Some of the disparity in the literature has been attributed to variations in the cell lines studied, ligand dose applied, methodologies utilized or model system evaluated (i.e. *in vitro* or *in vivo*). To evaluate the effects of heregulin on normal and malignant human breast and ovarian epithelial cells expressing known levels of the HER-2/ neu receptor, this report presents the use of several different assays, performed both *in vitro* and *in vivo*, *in vitro* proliferation assays, direct cell counts, clonogenicity under anchorage-dependent and anchorage-independent conditions, as well as the *in vivo* effects of heregulin on human cells growing in nude mice to address heregulin activity. Using a total of five different biologic assays in nine different cell lines, across two different epithelia and over a one log heregulin dose range, we obtained results that clearly indicate a growth-stimulatory role for this ligand in human breast and ovarian epithelial cells. We find no evidence that heregulin has any growth-inhibitory effects in human epithelial cells. We also quantitated the amount of each member of the type I receptor tyrosine kinase family (RTK I, i.e. HER-1, HER-2, HER-3 and HER-4) in the cell lines employed and correlated this to their respective heregulin responses. These data demonstrate that HER-2/neu overexpression itself affects the expression of other RTK I members and that cells expressing the highest levels of HER-2/neu have the greatest response to HRG.

Keywords: heregulin; NDF; HER-2; growth factor; epithelial cells

Introduction

A wide variety of growth factors bind to plasma membrane receptors found in human cells of diverse origin and serve to regulate cell growth as well as cellular activities other than mitogenesis (Sporn and

Roberts, 1988). A number of these peptide growth factors bind to the extracellular domains of a variety of receptor tyrosine kinases (RTKs), activating various signal transduction pathways (Aaronson, 1991).

Structural homologies of known oncogenes to some of these growth factors (GFs) or their cognate receptors indicate a potential role of these molecules in abnormal cell growth (Cross and Dexter, 1991). Alterations in either the structure or expression level of these genes can induce abnormalities in the control of cell proliferation, resulting in their putative pathogenic role in several human malignancies. The product of the HER-2/*neu* proto-oncogene is a 185 kD monomeric transmembrane tyrosine kinase (p185[HER-2/neu]) with extensive homology to both the epidermal growth factor receptor (EGFR) and the avian erythroblastosis virus oncogene, v-*erb*B (Coussens *et al.*, 1985; Prigent and Lemoine, 1992). Expression of the HER-2/*neu* proto-oncogene has been demonstrated in a number of normal human fetal and adult tissues, including breast and ovarian epithelia (Lemoine *et al.*, 1989; Natali *et al.*, 1990; Press *et al.*, 1990). Overexpression of the HER-2/*neu* receptor is found in 25–30% of human breast and ovarian cancers and is associated with a poor prognosis in those patients whose tumors contain the alteration (Slamon *et al.*, 1987, 1989; Press *et al.*, 1993). Studies directed at attempting to understand the activation and signal transduction pathways of HER-2/ *neu* have been limited until recently however, due to the lack of well characterized ligands that activate the p185[HER-2/neu] RTK. In the past few years, several putative ligands for the p185[HER-2/neu] receptor have been reported. Candidate ligands have been isolated from macrophages (Tarahovski *et al.*, 1991), bovine kidney (Huang and Huang, 1992), conditioned medium from transformed human T cells (Dobashi *et al.*, 1991), and rat transformed fibroblasts (Yarden and Peles, 1991), as well as human breast cancer cells (Lupu *et al.*, 1992). None of these molecules however were purified to homogeneity, cloned and/or sequenced, or recombinantly produced, making it difficult to study the specific interactions of these putative ligands with the HER-2/*neu* receptor. Identification, isolation and purification of specific activators of p185[HER-2/neu] made possible the simultaneous cloning of two homologous ligands; one from human breast cancer cells, heregulin (Holmes *et al.*, 1992), and the other from *ras*-transformed rat fibroblasts, *neu* differentiation factor (Peles *et al.*, 1992; Wen *et al.*, 1992). Despite the different cell source of heregulin (HRG) and *neu* differentiation factor (NDF), the proteins encoded by

these genes are identical at the amino acid level. The families of both HRG and NDF consist of α, β and γ isoforms (Holmes et al., 1992; Wen et al., 1992; Schaefer et al., 1997), with a total of 15 members described to date (Marchionni et al., 1993). Additional data indicate that both proteins induce phosphorylation of p185[HER-2/neu] after binding to receptor heterodimers which include HER-2/neu (Sliwkowski et al., 1994). Heregulin can directly bind p180[HER-3] and p180[HER-4] homodimers at low affinity, however, resultant phosphorylation only occurs with p180[HER-4] (Plowman et al., 1993; Carraway et al., 1994; Kita et al., 1994), while HER-2/HER-3 heterodimers constitute a high affinity receptor (Sliwkowski et al., 1994). In addition, formation of HER-2/HER-4 receptor heterodimers which can bind heregulin have been described (Karunagaran et al., 1996). These as well as other results, including downregulation of binding of EGF to EGFR by heregulin/NDF (Karunagaran et al., 1995), suggest that ligand binding can induce a series of complex interactions, resulting from heterodimerization and/or transphosphorylation of specific members of the RTK I family by other members of the group. The recent cloning of two new heregulin genes, neuregulin-2 (Carraway III et al., 1997; Chang et al., 1997) and neuregulin-3 (Zhang et al, 1997), that have different RTK I specificities, adds further complexity to this ligand-receptor system. The mechanism of type I receptor homo or heterodimerization resulting from HRG binding in human epithelial as well as neural cells is currently the subject of intense investigation. There is an evolving consensus indicating that the HER-2/neu receptor may play a critical role in heregulin-induced RTK I signal transduction (Carraway III et al., 1995; Pinkas-Kramarski et al., 1996; Lewis et al., 1996). The related biologic activities of HRG and HER-2 are supported by experiments in which these genes were either knocked out and/or mutated in mice, resulting in similar embryonic heart malformation and defects in the development of the neural system, causing embryonic death at day 11 (Meyer and Birchmeier, 1995; Lee et al., 1995). In addition, studies aimed at testing the effect of HRG on mammary gland development in vivo, mouse mammary epithelial cells cultured in matrigel, as well as mammary carcinoma tissue in explant culture, demonstrate response to addition of HRG by forming alveolar structures and this morphogenic effect is dependent on HER-2 activation of the MAPK kinase signaling pathway (Niemann et al., 1998).

Heregulin/NDF signaling has been associated with a number of diverse and at times seemingly contradictory biological activities. Heregulin has been reported to have a growth stimulatory effect on the HER-2/neu overexpressing breast cancer cell line SK-BR-3 (Holmes et al., 1992), as well as on HC11 mammary epithelial cells (Marte et al., 1995), mouse fibroblasts transfected with HER-2/neu and HER-3 (Carraway III et al., 1995) and 32D murine hematopoietic cells transfected with different RTK I combinations (Pinkas-Kramarski et al., 1996). Conversely, NDF has been reported as a growth inhibitory and differentiation-promoting factor in MDA-MB-453 and AU-565 mammary carcinoma cells (Peles et al., 1992; Bacus et al., 1993), as well as an inducer of apoptosis of breast epithelial cells (Daly et al., 1997). In addition, the in vivo role of heregulins as growth-stimulatory factors (GFs) is further suggested by reports of the cloning of genes encoding GFs with almost complete identity to HRG/NDF. These two molecules, acetylcholine receptor-inducing activity or ARIA (Corfas et al., 1993; Falls et al., 1993), and glial growth factor or GGF (Marchionni et al., 1993), activate phosphorylation of p185[HER-2/neu] and promote mitogenesis in cells of the peripheral nervous system. More recent data shows that GGF rescues peripheral nervous system cells from various apoptosis-induced conditions (Kopp et al., 1997; Raabe et al., 1997).

The putative pathogenic role of the HER-2/neu receptor in at least two major human malignancies (breast and ovarian cancer), and the controversy in the literature regarding the biologic effects of HRG/NDF on cells expressing p185[HER-2/neu], make further understanding of this receptor-ligand system important.

Table 1 Type I receptor quantitation

Cell line	HER-1 Receptors/cell	HER-2 Receptors/cell	HER-3 Receptors/cell	HER-4 Receptors/cell
Breast				
HMEC NEO	1140000	5260 ± 1570	283 ± 54	141 ± 103
HMEC H2	68100	210000	616	71
HBL-100 NEO	36000 ± 207	4450 ± 125	354 ± 119	BDL
HBL-100 H2	29000 ± 265	455000 ± 125	314 ± 105	BDL
MCF-7 PAR	4977 ± 193	15100 ± 73	24600 ± 797	360 ± 15
MCF-7 H2	BDL	1398945 ± 4394	150000 ± 249	296 ± 32
MDA-MB-231 NEO	201000 ± 1125	8090 ± 909	1410 ± 135	BDL
MDA-MB-231 H2	76000 ± 995	115000 ± 126	655 ± 63	BDL
MDA-MB-435 NEO	BDL	925 ± 14	7568 ± 25	BDL
MDA-MB-435 H2	BDL	28100 ± 371	11500 ± 392	BDL
SK-BR-3	40200 ± 157	1957800 ± 1860	12700 ± 135	201 ± 68
Ovarian				
2008 PAR	109000 ± 150	2460 ± 337	1020 ± 23	300 ± 95
2008 H2	202000 ± 36	104000 ± 339	769 ± 63	115 ± 16
C13 NEO	52500 ± 51	1260 ± 26	654 ± 34	195 ± 48
C13 H2	85100 ± 102	9100 ± 138	653 ± 30	175 ± 52
CaOv3 NEO	746 ± 58	5551 ± 232	5056 ± 285	68 ± 4
CaOv3 H2	BDL	104567 ± 9999	4863 ± 1500	115 ± 3
Detection limit	606	425	59	40

Receptor levels for all four members of the RTKI family in all the cell lines used were quantitated using a modified ELISA technique. Results reflect three independent quantitations, each performed in triplicate and represent the average number of receptors per cell, plus or minus the standard deviation. BDL: below detection limit

Most of the published reports to date have evaluated the effects of HRG/NDF on a single or a limited number of cell lines using a single assay method. To more fully evaluate the biological effects of HRG in a variety of human breast and ovarian cells we employed several different assays, as well as a number of cell lines with varying levels of HER-2/*neu* expression. This was done to circumvent the possibility that any observed effects might result from unique characteristics of an individual cell line or specific assay technique, as opposed to representing a more generic biologic response across epithelial cells. In addition, given the complex interactions reported between members of the RTK I family and HRG, we quantitated the expression levels of the four family members in each cell line studied and correlated these levels to the type and degree of HRG response. Subsequent evaluation of HRG's effects were tested *in vitro* using four different assays in a series of human breast and ovarian cell lines expressing defined levels of HER-2/*neu*, as well as *in vivo*, evaluating HRG effects on tumorigenicity in nude mice. We find evidence from both *in vitro* and *in vivo* experiments which support a growth-stimulatory, rather than a growth-inhibitory role for heregulin in human breast and ovarian epithelial cells. In addition,

we find that the expression level of each of the RTK I receptors (i.e. HER-1, HER-2, HER-3, HER-4) can profoundly effect HRG response, with HER-2 levels exhibiting the maximum association with growth stimulation.

Results

Quantitation of expression of receptor tyrosine kinases in human breast and ovarian epithelial cells

The effects of HRG on human breast and ovarian epithelial cells were evaluated using a panel of paired parental and HER-2 transfected cells, as well as HER-2 natural overexpressing cells (SK-BR-3). This pairing of parent/daughter cells with the same genetic background, allows us to isolate HER-2 expression as a variable and directly assess the biological consequences of its overexpression on HRG response.

To determine the relative effect of expression of the various members of the RTK I family on HRG response, a modified ELISA was used to quantitate the HER-1-4 receptor content of the various cell lines used in the *in vitro* and *in vivo* studies (Table 1). The
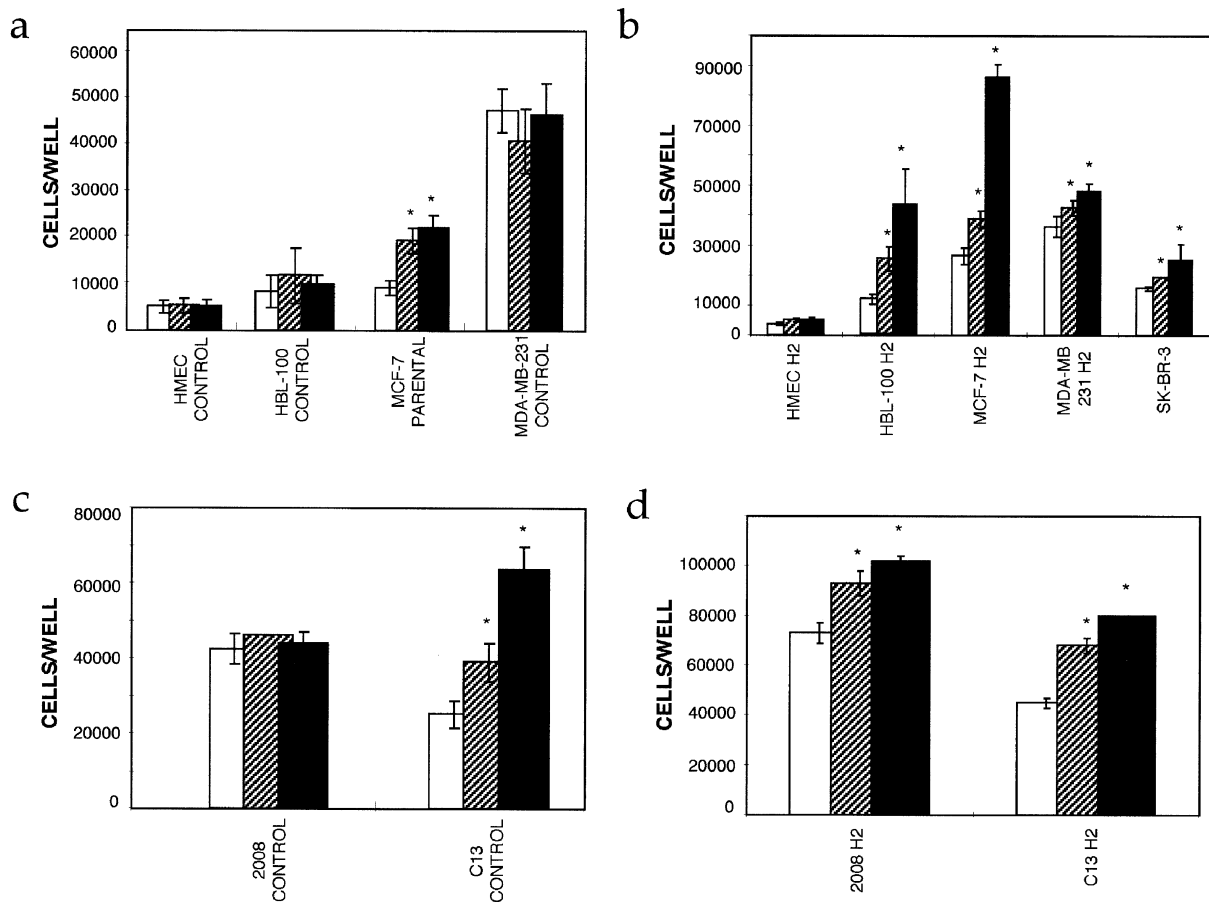

Figure 1 Effects of HRG on the proliferation of human breast and ovarian epithelial cells. Cells were seeded (10^3 total cells) in 6 well plates using $1 \times$ RPMI, containing 1% FBS as growth medium (5×10^3 total cells for the SKBR-3 cell line). After 24 h of growth at 37°C, cells were treated as indicated. Cell numbers were determined after 10 days using a hemocytometer. Cell lines in (**a** and **c**) represent normal, immortalized and malignant human breast and ovarian cells which contain a single-copy of HER-2/*neu* and express normal levels of the receptor. (**b** and **d**) illustrate the results obtained for human breast and ovarian cells containing multiple copies of HER-2/*neu* and overexpressing the receptor. Treatments included excipient control (☐), HRG at 1 nM (▨), or at 10 nM (■). Standard deviations are represented by bars at top of columns. Asterisks denote statistically significant *P* values (<0.01), when HRG and respective control treatment were compared

sensitivity of this ELISA was validated both by comparison with specific receptor content as determined by Scatchard analysis in a number of the cell lines (data not shown) as well as by quantitative comparison of the HER-2/neu expression levels in SK-BR-3 and MCF-7 cells. HER-2/neu expression levels have previously been shown to be 128 times higher in SK-BR-3 compared to MCF-7 cells by RNA analysis (Kraus et al., 1987; Paik et al., 1991). The ELISA data from the current study demonstrates a 129-fold increase in protein content when comparing these two cell lines which is almost identical to the published results and validating the ELISA results. HER-2/neu transfection results in cell lines with 10–100 times more HER-2 receptors/cell compared to matched non-overexpressing control cells (Table 1). This level of overexpression does not exceed the HER-2 level found in the SK-BR-3 cell line which is a natural, non-engineered, overexpressor of HER-2/neu. These transfection studies reveal that the number of receptors/cell for some RTK I members are altered by changes in HER-2/neu expression. Specifically, HER-1 (EGFR) expression is decreased and HER-3 expression is increased in 3 of 5 human breast epithelial cells engineered to overexpress HER-2, while human

ovarian epithelial cells tended to show the opposite pattern (Table 1). In addition, the number of HER-4 receptors/cell is considerably lower than those of the other RTK I receptors (Table 1). This result is not due to limitations in the sensitivity of the assay which can detect as little as 40 molecules/cell, but rather to the known low expression levels of HER-4 in breast cells (Pinkas-Kramarski et al., 1996).

Cell proliferation assays

After quantitation of the RTK I receptor levels in the human cell lines under study, growth assays were performed. Results from growth assays based on cell counts after long-term exposure to HRG in normal, immortalized and malignant human breast as well as malignant human ovarian epithelial cells are summarized in Figure 1a–d. Normal human mammary epithelial cells (HMEC RV/NEO), as well as immortalized HBL-100 cells do not demonstrate a growth-stimulatory or growth-inhibitory response to two different doses of HRG spanning one log (Figure 1a). However, overexpression of HER-2/neu in HBL-100 RV/H2 results in a dose-dependent growth-stimulatory response to HRG (Figure 1b). Similarly, this dose-
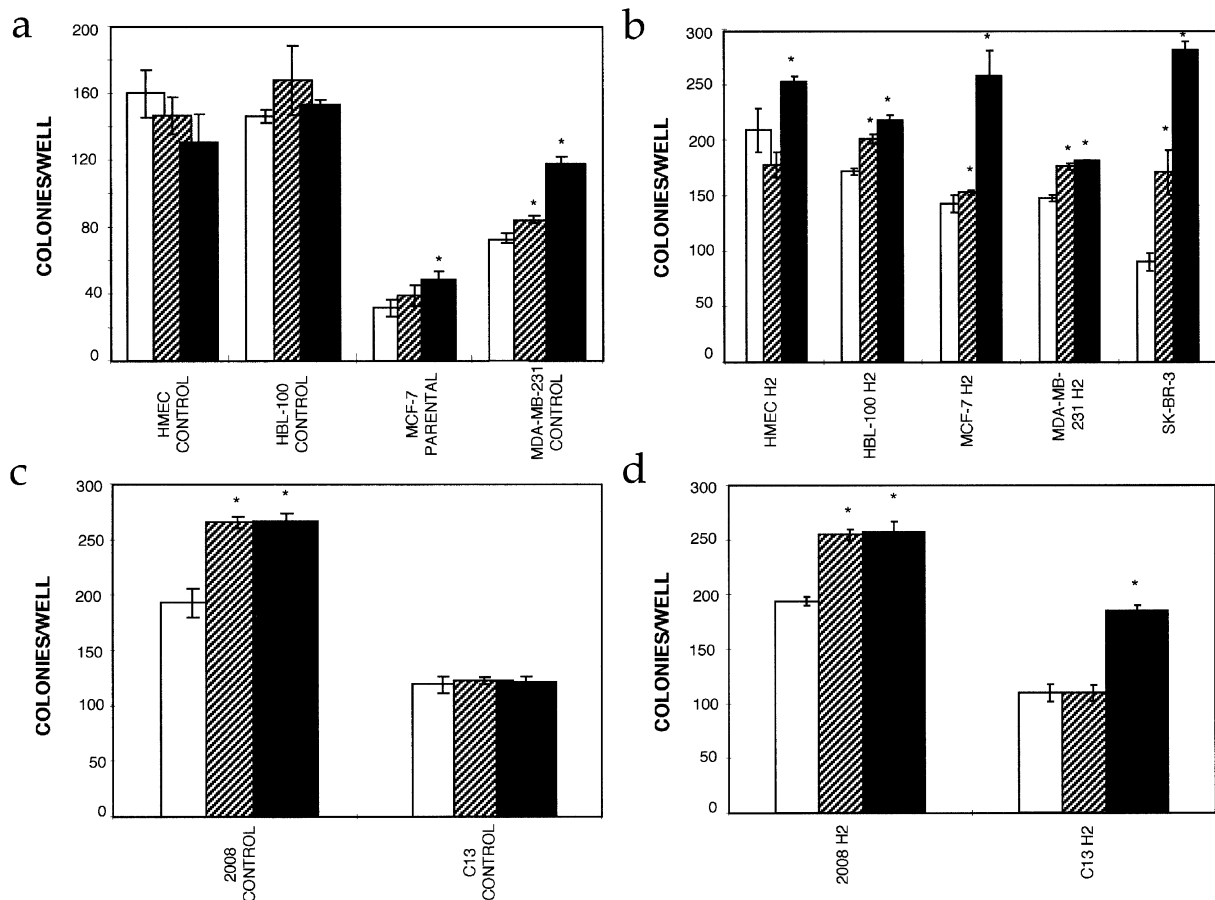

Figure 2 Effects of HRG on the clonogenicity of breast and ovarian epithelial cells. Cells were seeded (10^3 total cells) in 6 well plates using $1 \times$ RPMI, containing 1% FBS as growth medium (5×10^3 total cells for the SKBR-3 cell line in **b**). After 24 h of growth at 37°C, cells were treated as indicated. Total number of colonies per well after 10 days of incubation were counted after staining with hematoxylin. Cell lines in (**a** and **c**) represent normal, immortalized and malignant human breast and ovarian cells which contain a single-copy of HER-2/neu and express normal levels of the receptor. (**b** and **d**) illustrate the results obtained for human breast and ovarian cells containing multiple copies of HER-2/neu and overexpressing the receptor. Treatments included excipient control (☐), HRG at 1 nM (▨), or at 10 nM (■). Standard deviations are represented by bars at top of columns. Asterisks denote statistically significant P values (<0.01), when HRG and respective control treatment were compared

dependent growth stimulatory effect was seen both in malignant human breast and ovarian cancer cells which overexpress HER-2/neu (Figure 1b,d) demonstrating that cell lines overexpressing HER-2/neu consistently exhibit a statistically significant (P < 0.01), concentration-dependent growth response to HRG (Figure 1b,d). The two breast carcinoma cell lines expressing normal levels of the HER-2/neu receptor respond differently to HRG. The MCF-7 parental cells exhibit a dose-related growth-stimulatory response, while MDA-MB-231 cells are non-responsive (Figure 1a). A similar growth response to HRG treatment is observed for human ovarian cancer cells (Figure 1c,d). The HER-2 non-overexpressing 2008 parental cells do not respond to HRG treatment, while the non-overexpressing C13 parental cell line (a platinum resistant subclone of 2008) demonstrated a dose-dependent growth-stimulatory response to treatment. The variability in response underscores the need to evaluate more than one cell line to determine the biologic effects of HRG on non-overexpressing ovarian cells, but again demonstrates the consistent growth-stimulatory response in HER-2/neu overexpressing ovarian cancer cells. Statistical analysis comparing the growth response to HRG (HRG/XC) to the number of specific RTK I receptors/cell reveals a direct and linear correlation

between increased proliferation and the number of HER-2/neu receptors in breast cells and this phenomenon occurred at either the 1 nM (P = 0.001) or 10 nM (P = 0.0003) dose level.

Effects of heregulin on anchorage-dependent and independent growth of human breast and ovarian epithelial cells

Two different approaches were used to investigate the effects of HRG on anchorage-dependent and anchorage-independent growth in the same panel of human breast and ovarian cell lines. Clonogenic assays evaluate the anchorage-dependent capacity of cells to establish and form colonies when plated at low densities on plastic, while soft agar assays measure the anchorage-independent growth of cells in a semi-solid medium. The clonogenic effect of HRG on human breast and ovarian cells which do not overexpress the HER-2 receptor was variable (Figure 2a,c), however a statistically significant growth-stimulatory dose-response was again consistently observed when testing HER-2/neu overexpressing breast and ovarian cell lines (Figure 2b,d). Interestingly, cell lines exhibiting no response to HRG treatment in the *in vitro* cell growth assays (Figure 1) i.e., MDA-MB-231
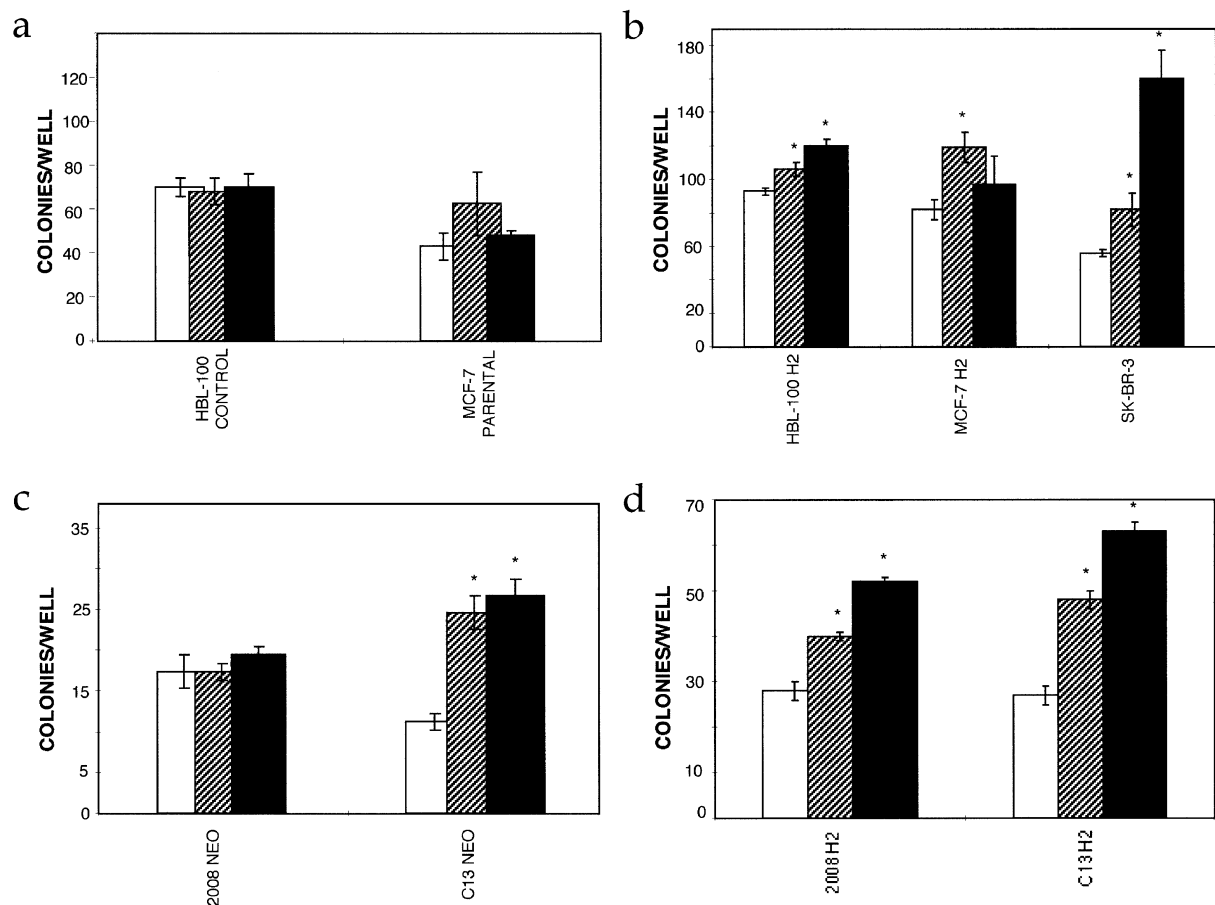

Figure 3 Effects of HRG on the anchorage-independent growth of breast and ovarian epithelial cells. Cells were grown (2×20^3) in 0.2% agar (in $1 \times$ RPMI, with 1% FBS). Cells were treated with the excipient control (☐), HRG at 1 nM (▨), or at 10 nM (■). The total number of colonies was counted after 3–4 weeks of incubation. Cell lines in (**a** and **c**) represent immortalized and malignant human breast and ovarian cells which contain a single-copy of HER-2/neu and express normal levels of the receptor. (**b** and **d**) contain the results obtained for human breast and ovarian cells containing multiple copies of HER-2/neu and overexpressing the receptor. Standard deviations are represented by bars at top of columns. Asterisks denote statistically significant *P* values (< 0.01), when HRG and respective control treatment were compared
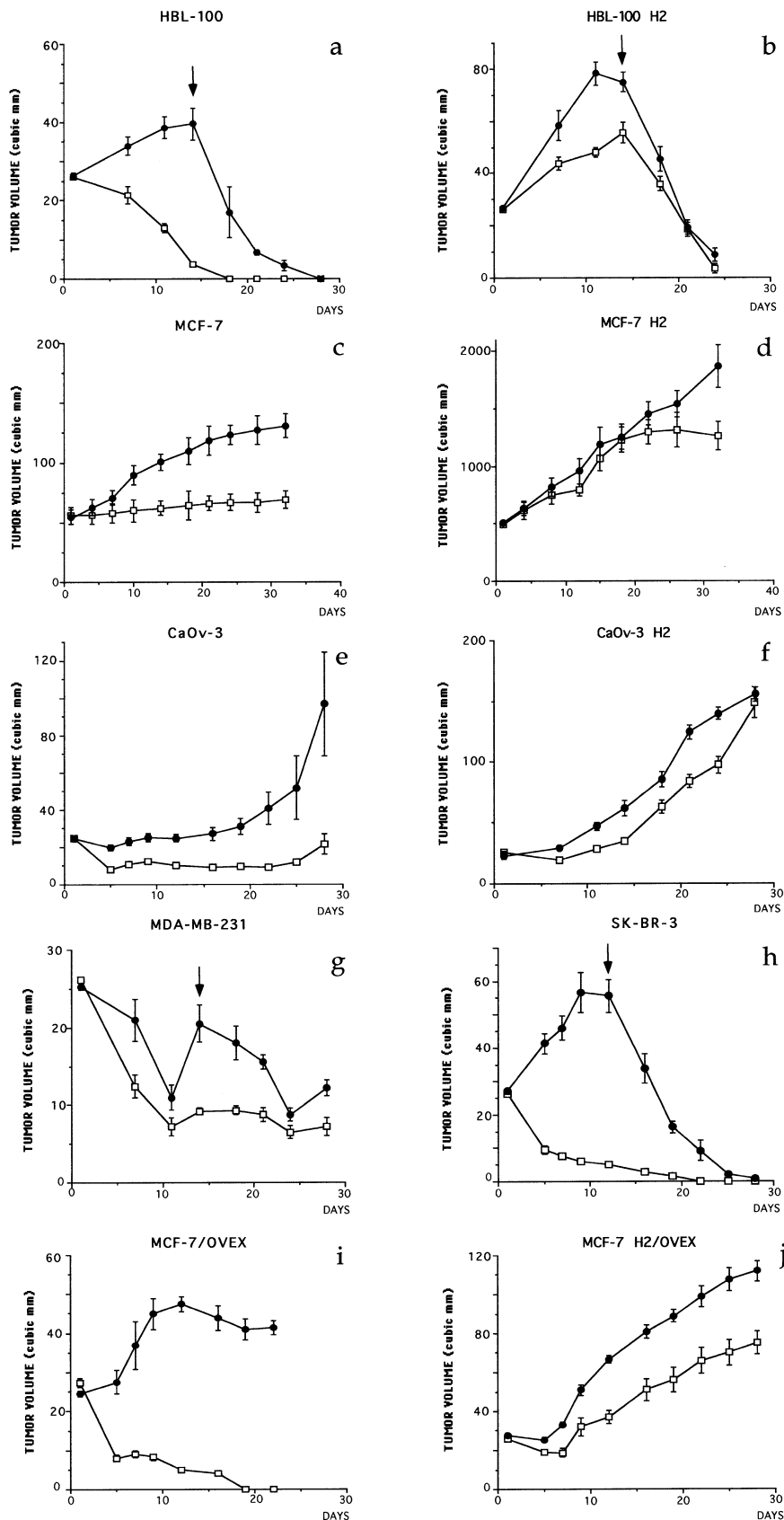
Figure 4 Effect of HRG on tumor formation in nude mice. Breast and ovarian cells expressing normal levels of HER-2 (**a, c, e, g** and **i**) or overexpressing HER-2/*neu* (**b, d, f, h** and **j**) were injected into nude mice. Estrogen support was provided by subcutaneous implantation of estrogen pellets 24 h prior to cell inoculation. Estrogen-deficient (ovariectomized) nude mice with no estrogen supplement were used (**i** and **j**). HRG (●) or the excipient control (□) were administered every other day starting 5–7 days after cells were implanted. Tumor volumes were measured at the indicated times. Arrows indicate the time at which HRG injection was stopped. Cell lines are as labeled in figure

and OV 2008, were responsive to treatment with HRG at both 1 and 10 nM in the anchorage-dependent assays (Figure 2a,c). Heregulin treatment results in formation of large diameter colonies compared to those of the normal human breast cell line HMEC, which generated very small colonies containing fewer cells (compare Figures 1a and 2a). This observation again underscores the point that the type of assay used can effect the result and may explain some of the variation in the published literature. Overall, the data from these studies indicate a profound stimulatory effect of HRG on the anchorage-dependent growth in all HER-2 overexpressing cells as well as some of the non-overexpressing cells.

Anchorage-independent growth assays conducted in semi-solid medium also demonstrated the capacity of HRG to promote in vitro colony growth in soft agar (Figure 3). All human breast and ovarian cell lines (both non-transformed and malignant) which over-express HER-2/neu again demonstrate a dose-dependent growth-stimulatory effect when tested in the semi-solid medium (Figure 3a – d). The only exception to this observation was the normal human breast epithelial cell line HMEC, which does not form colonies in semi-solid medium even with HER-2/neu overexpression (data not shown). Heregulin at a dose of 10 nM resulted in a decrease in the amount of colonies formed by MCF-7/H2 cells when compared to the 1 nM HRG dose. This does not however imply a growth inhibitory effect, since the number of colonies at the 10 nM dose was still greater than that obtained in the respective control wells. Regression analysis of response to HRG (HRG/XC) and RTK I receptor content again shows that the degree of responsiveness to HRG in both anchorage – dependent as well as anchorage – independent assays is linearly correlated with expression of the HER-2/neu receptor, with P

values of 0.00007 and 0.00002 for the 1 and 10 nM doses respectively.

[³H]thymidine incorporation assays were also performed and demonstrated HRG-induced increased counts (data not shown). The data from this panel of nine separate human breast and ovarian cell lines using two different doses of HRG and four different and independent in vitro cell growth assays, indicate a consistent and generic growth-stimulatory, rather than a growth-inhibitory effect for this ligand. These results support the initial data indicating a mitogenic activity of both HRG-α and HRG-β1 on cell lines over-expressing HER-2/neu (Holmes et al., 1992). Moreover, these results agree with the bulk of published literature showing that β forms of HRG are mitogenic (Lewis et al., 1996; Lu et al., 1995). In the present study we find no indication of a growth-inhibitory and/or differentia-tion effect, as previously reported by other investiga-tors working with NDF/HRG (Peles et al., 1992; Bacus et al., 1996).

In vivo tumorigenesis

The above results are restricted to the effects of HRG/ NDF on human breast and ovarian cancer cells in in vitro assays. Because in vitro response data may be more related to ex vivo growth conditions than generic biologic growth responses we decided to evaluate HRG effects in vivo. Tumorigenesis in nude mice is a widely accepted method of evaluating compounds for growth stimulation or inhibition of cells growing in vivo (Freshney, 1985). Results were obtained for the tumorigenic activity of HRG, as determined by implanting breast and ovarian cells subcutaneously into both non-estrogen primed and estrogen primed female nude mice and treating the animals with HRG (Figure 4). Cell lines were initially implanted into nude
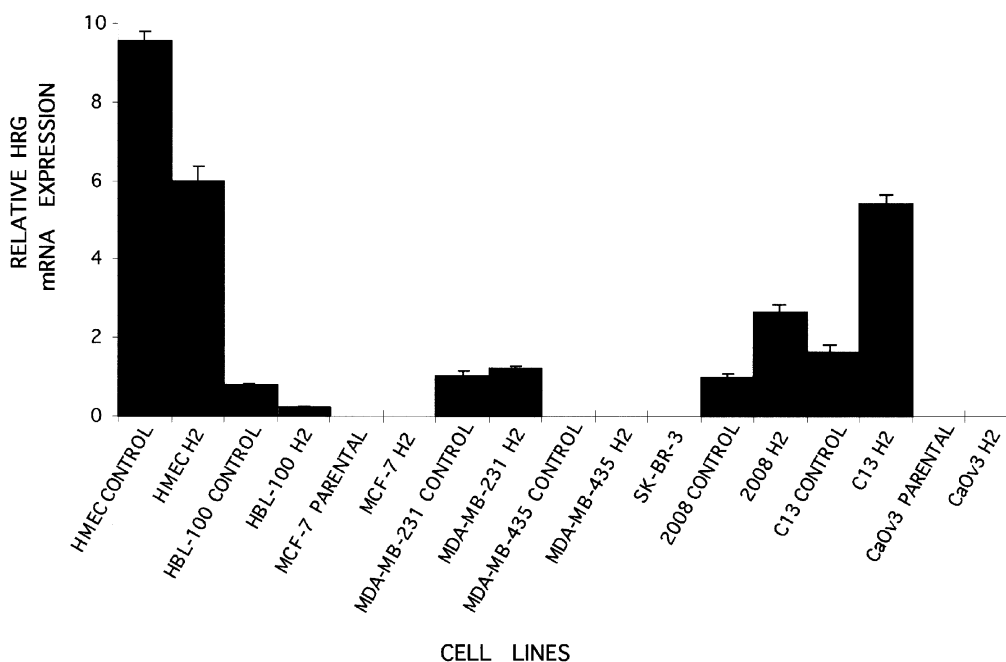

Figure 5 Heregulin production by human breast and ovarian cell lines. The quantitative RT – PCR technique Taqman was performed in triplicate using 100 ng of RNA per sample. HRG signal was normalized with the signal generated by a control gene, RPL19. Arbitrary units represent the average of triplicate numbers from the standard curve method. Bars at top of each column represent the standard deviation of each set of signal/sample

mice containing subcutaneous estrogen pellets (Figure 4a–h) or in ovariectomized mice with no external estrogen supplementation (Figure 4i,j). After establishment of tumors (5–7 days), animals were treated with rHRG-β1 or diluent control by subcutaneous injection. Comparison of *in vivo* growth of three different human breast and ovarian cell lines expressing normal levels of HER-2/*neu* (Figure 4a, c and e) as well as these same cells engineered to overexpress the gene (Figure 4b,d,f) demonstrates an increase in *in vivo* tumor formation induced by HRG treatment. A significant difference ($P = 0.0001$) was obtained for the growth of the immortalized breast cell line HBL-100 treated with HRG (Figure 4a) and this difference ($P = 0.0006$) was again observed in transformed, HER-2/*neu* overexpressing HBL-100 cells (Figure 4b). Tumors in mice implanted with MCF-7 parental cells and treated with rHRG-β1 were 1.6–2 times larger at day 15 ($P = 0.008$) and 3 times larger at day 32 ($P < 0.01$) when compared to mice injected with excipient control (Figure 4c). In addition MCF-7 cells engineered to overexpress HER-2/*neu*, formed tumors in both HRG-treated and the excipient control-treated groups, however tumor size in the heregulin treated group was again 1.6 times greater ($P < 0.002$) than in the control group by day 32 (Figure 4d). Larger tumors were also observed in HRG-treated mice implanted with non-overexpressing ($P = 0.02$) as well as overexpressing ($P = 0.03$) ovarian cancer cell line CaOv3 (Figure 4e,f respectively).

We also performed tumorigenicity assays for the estrogen-dependent MCF-7 non-overexpressing as well as the transfected HER-2 overexpressing matched cells in ovariectomized (estrogen-deficient) animals. These data demonstrate a significant ($P = 0.0001$ and $P < 0.01$ respectively) growth stimulatory effect of HRG on tumor formation for both cell lines in the absence of estrogen (Figure 4i and j). The data also demonstrate that MCF-7 cells overexpressing HER-2/*neu* are estrogen independent with respect to growth, but still respond to HRG supplementation (Figure 4j). The growth-stimulatory effect of HRG on cell lines growing *in vivo* was further evidenced when ligand injection was stopped. To determine the effects of endogenous production of HRG by implanted cells as well as the effects of natural (non-engineered) HER-2 overexpression on response to exogenous HRG, we evaluated the MDA-MB-231 and SK-BR-3 cells respectively (Figure 4g,h). The heregulin-producing malignant breast cell line MDA-MB-231 did not significantly form larger ($P = 0.07$) tumors in mice treated with HRG when compared to control-treated animals (Figure 4g). Conversely, SK-BR-3 cells which overexpress HER-2/*neu* but which do not produce endogenous HRG or grow in nude mice will form tumors with HRG treatment ($P = 0.0001$) (Figure 4h).

Quantitation of endogenous heregulin production by human breast and ovarian epithelial cells

Given the *in vivo* results obtained with the MDA-231 cells, it is possible that endogenous HRG production could effect response to exogenous HRG (Figures 1a and 4g, MDA-MB-231 cells). As a result we wished to determine if any of the other cells analysed in this study produced the ligand. All of the cell lines studied were analysed for HRG synthesis using a quantitative PCR technique called Taqman (Gibson *et al.*, 1996; Heid *et al.*, 1996) (Figure 5). The normal and immortalized human breast cell lines, HMEC and HBL-100, as well as their HER-2 transfected counterparts produce HRG. Conversely among the malignant breast cell lines, only MDA-MB-231 cells synthesize the ligand. Demonstration of HRG production by the MDA-MB-231 cells is consistent with previous data (Holmes *et al.*, 1992). MCF-7, MDA-MB-435 and SK-BR-3 cells make no detectable HRG. An interesting phenomenon occurs for the 2008 ovarian cancer cells and their platinum-resistant derivatives, C13 (Figure 5). Heregulin expression is consistently increased in these cells after HER-2 transfection and overexpression. The opposite phenomenom was observed for the normal and immortalized breast cell lines HMEC and HBL-100 respectively, where a reduction of HRG expression is observed with HER-2 overexpression. These results were confirmed by Northern blot analysis (data not shown). The malignant ovarian human cell line CaOv3 and its HER-2 overexpressing transfectant however do not make the ligand.

Discussion

HER-2/*neu* is a member of the type I receptor tyrosine kinase family (RTK I) of epithelial growth factor receptors. Transfection and overexpression of this gene confers a growth advantage and induces transformation of a number of non human, mammalian cells *in vitro* (Hudziak *et al.*, 1988; Chazin *et al.*, 1992). In addition, HER-2 overexpression resulting from gene amplification occurs in 25–30% of human breast and ovarian cancers and this overexpression is associated with poor prognosis in those patients whose cancers contain it (Slamon *et al.*, 1987, 1989; Press *et al.*, 1993). The potential pathogenic role of overexpression of this growth factor receptor in two major human malignancies has made understanding the biologic effects of its activation the subject of intense investigation. Many growth factors are capable of displaying mitogenic, differentiation or growth inhibitory effects on target cells (Sporn and Roberts, 1988). Pleiotropic response to peptide growth factors assayed under different conditions is a well documented phenomenom (Sporn and Roberts, 1988). The objective of this study was to evaluate biologic effects of heregulin on human breast and ovarian epithelial cells expressing defined levels of the HER-2/*neu* receptor, spanning the spectrum from normal levels to the overexpressed levels found in cells with HER-2/*neu* amplification. The isolation, purification and cloning of heregulin (Holmes *et al.*, 1992), an activator of RTK Is including HER-2/*neu*, makes investigation of the biologic effects of this ligand possible. In this study a variety of growth assays were used to evaluate a large number of human breast and ovarian epithelial cells growing both *in vitro* and *in vivo* across a one log concentration of HRG. This approach was undertaken to circumvent the possibility that biologic effects which are unique to a specific cell line, method of analysis or dose level, might lead to incorrect conclusions as to the generic effects of this ligand. The results obtained using this approach provide a consistent and reproducible picture of HRG response in human breast and ovarian epithelial

cells. The data demonstrate a varying growth response to HRG in cell lines containing a single copy of the HER-2/neu gene and expressing normal amounts of the gene product. The immortalized breast epithelial cell line, HBL-100, as well as malignant breast cell lines MDA-MB-231 and MCF-7 all contain a single copy of HER-2/neu gene and express normal levels of the protein (Press et al., 1993). The non-amplified, non-overexpressing MDA-MB-231 and HBL-100 cells do not respond to HRG treatment, while MCF-7 cells exibit a growth-stimulatory response. Examination of RTK I levels in these cell lines demonstrates that the MCF-7 cells express greater levels of HER-2 (1.9–3.4-fold) and HER-3 (17–69-fold) than those found in HBL-100 and MDA-MB-231 cells. This phenomenon could account for the differences in growth response, since HER-2/HER-3 heterodimers are known to be important in the signaling response associated with HRG (Riese et al., 1995; Beerli et al., 1995). This is further supported by the lack of responsiveness of the normal breast epithelial cell line HMEC, which contains relatively low levels of HER-3 receptor. An alternative explanation for this difference could relate to the fact that both the MDA-MB-231 and HBL-100 cell lines produce HRG endogenously, while MCF-7 cells do not. The endogenous production of this ligand may stimulate cell growth via an autocrine mechanism (Schaefer et al., 1997), lessening the effects of exogenously added HRG. With the single exception of HMEC cells, transfection of HER-2/neu and its concomitant overexpression results in a growth-stimulatory response to HRG in the in vitro growth proliferation assay in all breast and ovarian cell lines examined, regardless of endogenous HRG expression. Additionally, in the current study we demonstrate that treatment with HRG results in further increased growth of cells that overexpress HER-2/neu.

Results of the anchorage-dependent assays also demonstrated a correlation between overexpression of the HER-2/neu receptor and a HRG-induced growth stimulatory and clonogenic responses in both normal and malignant breast epithelial cells as well as ovarian cancer cells. It is interesting to note that the malignant breast cell line MDA-MB-231, which do not show a response to HRG in the cell proliferation assay, display a significant concentration-dependent increase in colony formation in the anchorage-dependent growth assay underscoring the need to evaluate biological responses by more than one assay. The linear correlation coefficients obtained between anchorage-dependent growth and HER-2 receptor number again demonstrates a relationship between HER-2 expression and response to heregulin and further supports the concept that HER-2/neu is an important mediator of HRG-induced clonogenic growth activity in human breast and ovarian cells. The results also support a role for HRG as a survival factor as reported by investigators working with neural derived cells (Koop et al., 1997; Raabe et al., 1997), since cells plated at low density and in low serum, i.e. clonogenic conditions, not only survived but were growth-stimulated by the ligand. Finally, in experiments aimed at detecting the possible effects of HRG on apoptosis, MCF-7 parental, MCF-7 RV/H2 and SK-BR-3 cells were stained for Anexin V after 3, 5 and 10 days of incubation (data not shown). These studies failed to confirm induction of apoptosis by HRG in malignant breast cells as reported by others (Daly et al., 1997). Results from soft agar assays further confirm the stimulatory effects of HRG on anchorage-independent growth. Immortalized, nontransformed as well as malignant breast, and ovarian epithelial cell lines overexpressing HER-2/neu demonstrate a marked increase in soft agar growth in response to HRG exposure.

Tumorigenicity of both parental and HER-2/neu transfected cells in nude mice treated with HRG was consistently higher when compared to the growth observed in untreated mice. Only the MDA-MB-231 breast cancer cells were unaffected by treatment with HRG, perhaps due to endogenous HRG production by this cell line. In vivo HRG treatment of ovariectomized mice implanted with the estrogen-dependent MCF-7 parental cells yielded interesting results. As expected no tumor growth was observed in control mice, however tumors did form with HRG treatment. These results suggest that HRG is able to overcome at least some of the estrogen-dependency of MCF-7 cells in vivo and further substantiate the role of this ligand as a growth-stimulatory factor. Previous studies from our laboratory have shown direct interactions between the estrogen receptor and p185$^{HER-2/neu}$, suggesting a mechanism for estrogen-independence in HER-2/neu overexpressing breast cancer cells (Pietras et al., 1995).

Previously published studies have reported a variety of different and sometimes opposite effects of HRG/NDF on human breast epithelial cells. Heregulin/NDF has been reported to induce growth inhibition and/or cell differentiation (Lupu et al., 1992; Peles et al., 1992; Bacus et al., 1996) as well as apoptosis (Daly et al., 1997) in some cell lines. Conversely, growth stimulation by HRG has been reported in other cells (Holmes et al., 1992; Marchionni et al., 1993; Carraway III et al., 1995; Levi et al., 1995; Marikovsky et al., 1995; Pinkas-Kramarski et al., 1996; Marte et al., 1995; Lewis et al., 1996). In general these reports have evaluated a limited number of cell lines and/or used a specific in vitro assay as well as a fixed concentration of HRG/NDF. Some reports in which treatment of cells with NDF resulted in growth inhibition or cell differentiation used the MDA-MB-453 and AU-565 cell lines (Bacus et al., 1993; Daly et al., 1997). We did not evaluate these two cell lines in this study; however, other investigators have previously reported growth stimulation of MDA-MB-453 cells when treated with the same doses of HRG in 1% FBS (Lewis et al., 1996). Moreover, the AU-565 cells are derived from the same patient as SK-BR-3 cells, sharing a common origin (Bacus et al., 1993). Similarly use of in vitro assays alone to characterize biological responses to a ligand can be misleading. An example of this is the fact that the initial published literature on the effects of high dose EGF demonstrated a growth inhibitory response, however subsequent studies demonstrated EGF to be clearly growth-stimulatory in vivo (reviewed in Carpenter and Cohen, 1990; Khazaie et al., 1993). Further evidence supporting the growth stimulatory activities of HRG/NDF on breast cancer cells is found in two recent reports in which the effects of NDF were studied in two different in vivo models. In the first, NDF expression induced formation of adenocarcinomas (Krane and Leder, 1996). In the second, transfection

of the human breast cancer cell line MDA-MB-435 with either scatter factor (HGF) or HRG/NDF increased tumor size at the primary site and induced metastasis of transfected cells to the lungs (Meiners *et al.*, 1998).

Binding of heregulin to two different members of the RTK I family, specifically HER-3 (Sliwkowski *et al.*, 1994), and HER-4 (Plowman *et al.*, 1993), as well as the documented interactions between these receptors and EGFR (Prigent and Lemoine, 1992; Karunagaran *et al.*, 1995, 1996; Pinkas-Kramarski *et al.*, 1996; Riese *et al.*, 1995; Spivak-Kroizman *et al.*, 1992; Qian *et al.*, 1994; Alimandi *et al.*, 1997), suggests that complex combinations of receptor-ligand interactions are occurring in human cells expressing these receptors. Additionally, binding of EGF to HER-2/HER-3 heterodimers occurs (Alimandi *et al.*, 1997), indicating that competition between HRG and EGF for binding to this receptor combination might occur *in vivo*. The current results indicate that expression of all four RTK I receptors varies significantly from cell to cell and that HER-2 receptor number can significantly effect expression of the other RTK I members. This report also indicates that biological responses to heregulin both *in vitro* and *in vivo* are directly correlated with HER-2/*neu* expression levels. This confirms results from other investigators indicating that heregulin signaling through HER-3 and HER-4 may be dependent on HER-2/*neu* expression levels in breast (Riese *et al.*, 1995; Beerli *et al.*, 1995) as well as prostate cells (Leung *et al.*, 1997; Grasso *et al.*, 1997). The observed variability for the expression of the different RTK type I, in particular the changes in HER-3 number post HER-2 transfection, most likely have an impact on the extent of the effect of HRG. This could explain the observed range in growth stimulatory responses resulting from treatment with heregulin. Results in the current study however, do not support a significant role for HER-4 as an important molecule in the growth stimulatory HRG-induced effects in human breast and ovarian epithelial cells.

There are published reports of differentiation induced by NDF in breast cancer cells growing in high fetal bovine serum (FBS) concentrations (Peles *et al.*, 1992). These reports, however, studied changes in expression of casein and milk fat globulin which are markers of mammary cell maturation rather than tumor cell differentiation (Stampfer and Yaswen, 1999). It should be noted that normal breast epithelial cells show a high degree of phenotypic plasticity in monolayer and express many features of malignant cells *in vivo* (Barcellos-Hoff *et al.*, 1989; Petersen *et al.*, 1992). In addition, the use of conditioned medium and FBS introduces variables due to the presence of other growth regulatory activities in serum which make it difficult to evaluate the effects of a specific growth factor, i.e. HRG/NDF, on cell growth. Indeed previous reports have demonstrated that study of the behavior of transformed cell lines growing in monolayer cultures may be less useful in assessing the activity of a specific growth factor on cell differentiation (Petersen *et al.*, 1992). All assays in the current study were performed using 1% FBS in the culture medium. Lowering the concentration of serum in the culture medium should diminish the effects of other serum GFs which activity could mask, or alter HRG effects on the cell lines

(Lewis *et al.*, 1996). A potential difficulty in lowering serum concentration to 1% is that itself can inhibit cell growth. This did not occur with the cell lines used in this study (Figures 1–3, see Lewis *et al.*, 1996). Published reports on the effects HRG/NDF activity have also been confounded by the use of different recombinant forms of the ligand that span overlapping, but not identical regions of the molecule and/or by the use of different isoforms of HRG which are reported to promote activation of different receptor combinations (Pinkas-Kramarski *et al.*, 1996; Weiß *et al.*, 1997).

Given the putative role of HER-2/*neu* in the pathogenesis of human breast and ovarian cancer, characterization of the nature and biologic effects of ligands which either directly or indirectly interact with this receptor are of potential significance. The current study demonstrates that a number of human breast and ovarian cells expressing normal levels of HER-2 are growth-stimulated by HRG and that when these same cells overexpress the receptor, growth stimulation is a uniform response. A more complete understanding of the growth activity signals generated through HER-2/*neu* as well as other RTK I family members could have important therapeutic implications in human breast and ovarian cancers.

Materials and methods

Cell lines

Normal human mammary epithelial cells (HMEC) were purchased from Clonetics (San Diego, CA, USA) and grown as directed by the supplier. Immortalized human breast cells (HBL-100), and the breast carcinoma cell lines MCF-7, MDA-MB-231, MDA-MB-435, SK-BR-3 as well as the ovarian malignant cell line CaOV3 were obtained from the ATCC (American type culture collection). The ovarian cell line 2008 and its platinum-resistant subclone C13 were kindly provided by Dr Steven Howell (University of California, San Diego, USA). All cell lines with the exception of HMEC were routinely maintained in RPMI medium (Gibco), containing 10% heat inactivated fetal bovine serum (FBS), penicillin and streptomycin. Non-tranfected, parental cell lines, are designated by the suffix PAR. HER-2/*neu* overexpressing cell lines designated by the suffix /H2, and the mock transfected cell lines (/NEO) were generated by multiple infections of cells with a replication defective retroviral vector containing a full-length human HER-2 cDNA with a neomycin resistance gene and control cells were transfected with the vector containing the neomycin gene alone as described (Chazin *et al.*, 1992).

Cell proliferation assays

Cells were plated in six well plates (four plates/cell line) at the desired cell density (1000 cells/well or as otherwise indicated) in RPMI containing 1% FBS. After incubation at 37°C with 5% CO_2, for 24 h, the 7 kDa rHRG–β1 ligand, or control solution (0.1% Trifluoroacetic acid, 30% CH_3CN) were added at two different doses to the plates (1 and 10 nM final concentration in a total volume of 3 ml). Cells were then incubated at 37°C with 5% CO_2 and after 10 days of growth, half of the plate (three wells) was used for direct cell counts by trypan blue exclusion, and half was used for colony counts (see clonogenic assay below). All *in vitro* assays were performed in triplicate two to three times. Results obtained were analysed using the Mann Whitney *U*-non-parametric statistical test (Stat View 4.5, Abacus concepts; Berkeley, CA, USA).

Anchorage dependent and independent growth

Clonogenic assays were performed as described above on plastic to measure anchorage-dependent ability of cells to form colonies when plated at low densities (Paraskeva *et al.*, 1990). The total number of colonies/well/plate was determined by staining with hematoxylin using standard cytochemical techniques. Quantitation of colonies in soft agar was used to measure anchorage-independent growth. Using a 0.3 ml bottom layer of 0.4% agar (Collaborative Research) in 1% FBS RPMI formed in the eight central wells of a 24 well plastic dish. After solidification of the bottom layer, a top layer containing a total of 2000 cells and the desired concentrations of either diluent control or 7 kD rHRG-β1 (1 and 10 nM) in 0.3 ml of 0.2% agar in $1 \times$ RPMI was layered on top. Colonies were stained with 0.2 mg/ml tetrazolium violet (Sigma, St Louis, MO USA) in PBS after 2–4 weeks and quantitated by direct observation with an inverted microscope (Diaphot, Nikon, Japan).

Tumorigenesis

Tumor formation by cells expressing low and high levels of HER-2/*neu* were determined after implantation of cells in female nude mice ($n = 8$ *per* treatment group) either with or without estrogen supplementation (1.7 mg pellet subcutaneously). Recombinant heregulin-β1 was injected at a dose of 2 mg/kg subcutaneously every other day. Tumor size was measured in three dimensions with micrometer calipers. Tumor measurements collected at each time point were compared using a two way statistical analysis of variance throughout the course of the experiment (unless otherwise indicated) using Super ANOVA and StatView 4.5 (Abacus concepts; Berkeley, CA, USA) programs.

Preparation of cell lysates for receptor quantitation

Cells were grown to approximately 90% confluence and harvested using PBS with 2 mM EDTA then counted and stored as frozen cell pellets. Lysates were prepared by thawing and resuspending the cells in 25 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1.0% v/v Triton X-100, 1.0% w/v CHAPS, 10% v/v glycerol (lysis buffer) with 0.2 mM PMSF, 10 KIU/ml aprotinin, 10 μM leupeptin and 16 μM bestatin.

RTK I receptor quantitation

RTK I receptor (HER 1, HER-2, HER-3 and HER-4) expression levels for each cell line used in this study were determined using a receptor specific enzyme linked immunosorbent assays (ELISA). The assays were configured in a sandwich format in 96-well plates (Immulon 4, Dynatech) coated with receptor specific monoclonal antibodies by incubating them with purified specific anti-RTK I antibodies, diluted to 1.0 μg/ml in 50 mM carbonate, pH = 9.6, overnight at 4°C. Monoclonal antibodies 13A9, 2H11, 2F9, and 1E3, (Genentech, Inc. South San Francisco, CA, USA) were used for the EGFR, HER-2, HER-3, and HER-4 assays, respectively. These antibodies recognize specific epitopes within the extracellular domains of their corresponding receptors. The following day ELISA plates were blocked with 2.0% w/v BSA in 25 mM Tris, pH = 7.5, 150 mM NaCl, 0.02% v/v Tween 20 (TBST) for 1 h at room temperature. Cell lysates were added to the MAb coated plates and incubated for at least 1 h at room temperature. The plates were then washed with TBST and incubated with polyclonal detection antibodies. Rabbit polyclonal antibodies (Santa Cruz) specific for cytoplasmic domain epitopes were used for the HER-1, HER-3 and HER-4 assays. A guinea pig polyclonal antibody (Genentech) raised against the extracellular domain of HER-2 was used for the HER-2 assay. After 1 h, plates were washed with TBST and incubated with anti-primary-biotinylated, then with streptavidin-HRP, followed for an additional incubation with a streptavidin-peroxidase conjugate (Calbiochem). Following a final wash with TBST the plates were developed using a pre-formulated o-phenylenediamine substrate tablet (Sigma). A purified recombinant fragment of HER-2, corresponding to the extracellular domain of the full-length receptor, was used to standardize the HER-2 ELISA. Serial dilutions of cell lysates containing known receptor concentrations were used to generate standard curves for the HER-1, HER-3, and HER-4 ELISA's. K562 transfectants were used for the HER-3 and HER-4 standard lysates, and MDA 468 cells were used for the HER-1 standard lysate. Validation of the ELISA assay was performed by determining receptor content using Scatchard analysis of receptor content (Munson and Rodbard, 1980) in competitive ligand binding experiments (Holmes *et al.*, 1992). The limit of sensitivity for each assay was set at 10% of the basal signal for the standard lysate curves and the detection limits expressed in molecules/cell for each receptor were 606, 425, 59 and 40 for HER-1, HER-2, HER-3 and HER-4 respectively. Regression analyses were performed using StatView 4.5 (Abacus concepts; Berkeley, CA, USA).

Real time quantitative PCR (TaqMan)

Heregulin mRNA was quantitated in each cell line using RT–PCR or TaqMan as previously described (Gibson *et al.*, 1996; Heid *et al.*, 1996). The sequence of the primer/probe set, specific to the EGF domain of HRG, used in this analysis are shown below: F, 5′-TGTGCGGAGAAGGAGAA-AACTTTCT-3′; R, 5′-GTTGGCACTTGCACAAGTATC-TCG-3′; P, 5′-FAM-CTTACAAGCCGCATCGATTTTGT-C-TAMRA-p-3′; where F and R are the forward and reverse primers respectively, and P is the flourescent labeled probe. Ribosomal protein L19 (RPL19) was used as the housekeeping gene. Primer/probe sets for RPL19 are: F, 5′-ATGTATCACAGCCTGTACCTG-3′; R, 5′-TTCTTGGT-CTCTTCCTCCTTG-3′; P, 5′-FAM-AGGTCTAAGACC-AAGGAAGCACGCAA-TAMRA-p-3′.

TaqMan analysis was performed in a standard 96-well plate format using an ABI PRISM 7700 Sequence Detection System instrument and software (PE-Applied Biosystems, Inc). Standard curves were constructed using 0.8–200 ng total RNA isolated from the breast cell line MDA-MB-231 for HRG and 0.3–75 ng for RPL19. Each dilution was run in duplicate. For samples derived from the respective cell lines 100 ng were analysed in triplicate for HRG and RPL19.

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