Heregulin Is Sufficient for the Promotion of Tumorigenicity and Metastasis of Breast Cancer Cells \textit{in Vivo}

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Abstract

Resistance of breast carcinomas to hormonal therapy is a clinical obstacle for the treatment of breast cancer. The molecular mechanisms and the factors involved in the progression of tumors from an estrogen (E2)-dependent to an E2-independent phenotype are not entirely understood. Heregulin (HRG) is a pleiotropic growth factor that binds to the erbB family of receptors, which are correlated with breast cancer progression and an aggressive phenotype in the breast carcinomas overexpressing the receptors. Previous studies in transgenic mice have shown that HRG is sufficient to induce mammary gland transformation and proliferation in the presence of hormonal stimulation. However, these studies did not address the important issue of the E2 independence that is part of the progression of breast cancer. In this study, we investigated the role of HRG in E2 independence. We were able to determine that HRG up-regulation was sufficient for the development of mammary tumors in the absence of E2 stimulation, a situation that mimics the progression of the human disease. We demonstrated that in ovariectomized nude mice, HRG induced E2 independence and antiestrogen resistance and promoted metastasis and preneoplastic transformation of the adjacent mouse mammary tissue. We show that one of the mechanisms by which HRG achieves the aggressive phenotype may be mediated via an increase in activated mitogen-activated protein kinase, an increase in a matrix-degrading enzyme, MMP-9, and the overexpression of vascular endothelial growth factors. The up-regulation of these genes occurred in the absence of any additional stimulation, in an autocrine manner. Our data provide new insights into the mechanisms of breast cancer progression \textit{in vivo}, and reinforce the important role that HRG plays in this process.

Introduction

About 60\% of the human breast carcinomas express estrogen receptors (ERs) and are characterized by a better prognosis and response to endocrine treatment (1). Unfortunately, at some point, most of the initially responsive patients will fail the endocrine treatment and will develop more aggressive tumors. ER expression is important in predicting the response to adjuvant hormone therapy, although its role as a prognostic indicator is not clear (2).

The growth and progression of breast carcinomas are regulated by a milieu of signals mediated by growth factors and steroid receptors. The more invasive phenotype of breast cancer has been correlated with up-regulation of \textit{erb}B2, a marker for poor prognosis. Several studies have suggested a negative correlation between the \textit{erb}B2 and the estrogen (E2) pathways. E2 inhibits \textit{erb}B2 overexpression and tamoxifen (Tam) up-regulates \textit{erb}B2 in ER-positive (ER+) human breast cancer cells (3). Clinical and experimental data suggest that co-expression of the \textit{erb}B2 oncoprotein in ER+ breast cancer cells confers resistance to endocrine therapy. In fact, the response rate to Tam, shown to be around 50\% in ER+ patients, is reduced to 17\% in the presence of \textit{erb}B2 overexpression (4). An interesting inverse correlation has also been found between overexpression of \textit{erb}B2 and loss of functional ER (5). The decrease in the responsiveness of breast carcinomas to E2 has been linked to the activation of the \textit{erb}B2 receptor via overexpression or binding of heregulin (HRG) to its receptors (6). HRG (initially called gp30) (7) and its rat homologous Neu erbB4 (9). HRG antagonizes the E2-mediated down-regulation of \textit{erb}B2 and is capable of enhancing Tam-induced stimulation of the receptor (6). Recent \textit{in vitro} data from our laboratory have shown the loss of E2 dependence and the acquisition of Tam resistance following the transfection of HRG(2) cDNA into MCF-7 cells, an E2-dependent breast cancer cell line (10). Treatment of breast cancer cells with HRG on the background of high \textit{erb}B2 expression results in ER phosphorylation and up-regulation of progesterone receptor (PR) expression (11). This acquired phenotype depicts the passage from an initial E2-responsive and antiestrogen-sensitive tumor cell to a later step in carcinogenesis, resembling the common progress of many human breast tumors. The molecular mechanism for this

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process is not entirely understood. Recent data suggest that HRG mediates active repression of E2 response elements via up-regulation of metastasis-associated protein 1 (MTA-1). Physical interaction between MTA-1, the activation factor domain of ERα, and histone-deacetylase 1 and 2 has been reported, implicating MTA-1 as a repressor of gene expression and a downstream effector of HRG (12).

The angiogenesis process is an essential requirement for tumor progression and for the survival of solid tumors (13). Growth factors known to mediate this process belong to the vascular endothelial growth factor (VEGF) family. Overexpression of VEGF has been detected in tumor specimens and correlated with an advanced invasive phenotype. The expression of these factors is up-regulated via erbB2 activation and the ras pathway (14, 15). Recent data from *in vitro* studies have implicated HRG in the angiogenesis and invasion processes. HRG promoted an invasive phenotype of breast cancer cells *in vitro* (10), and on treatment of breast cancer cells with HRG, up-regulation of VEGF at the mRNA and protein levels was observed (10). This effect was blocked by the anti-erbB2 receptor monoclonal antibody herceptin and was dependent on functional p21-activated kinase-1 (16). An interesting finding was that this up-regulation could occur independently of erbB2 expression, suggesting an alternative mechanism for the HRG-induced increased levels of VEGF (17).

Reduced cell adhesion, increased migration, and increased secretion of both soluble and membrane-associated degrading proteinases are invasion-associated processes influenced by oncopgenes, growth factors, steroid hormones, and the extracellular matrix (ECM) (18, 19). MMPs are a family of more than 11 zinc-dependent endopeptidases (20) that increase invasiveness (21). One such enzyme is MMP-9 or gelatinase B, which is highly expressed in many human malignancies including breast carcinomas (22). Metastasis induced by activated ras-transformed cells has been correlated with MMP-9 release (23, 24). ProMMP-9 is up-regulated in breast tumors as compared to noncancerous tissue (25).

In this study, we demonstrate that HRG is a tumor-promoting factor using a unique *in vivo* model system of breast cancer tumor progression. HRG promotes the *in vivo* progression, from an E2-dependent, antiestrogen-sensitive and non-metastatic phenotype to the E2-independent, antiestrogen-resistant, and metastatic phenotype. We show that the possible secretion of HRG from the localized tumors induces the preneoplastic transformation of the mouse mammary gland. Our *in vivo* model offers an interesting and useful insight into the understanding of possible factors implicated in breast cancer progression and resistance to hormonal therapy. Our pathological findings also support the effect of secreted HRG on the surrounding tissue. This is the demonstration that constitutive expression of HRG, as opposed to cells treated with the growth factor, regulates the expression of VEGF *in vivo*. These findings are of great relevance to the human disease, where up-regulation of VEGF has been positively correlated with the expression of breast carcinomas. We also demonstrate that the mechanism of HRG action in promoting the progression is associated with an increase in mitogen-activated protein kinase (MAPK) phosphorylation, which implicates the ras-signaling pathway, and an increase in activated MMP-9.

### Results

#### HRGβ2 Induces E2-Independent and Antiestrogen-Resistant Tumors in Ovariectomized Athymic Nude Mice

The cells MCF-7 (parental), MCF-7/V (vector), and MCF-7/HRG (HRG transfected) were inoculated in the mammary fat pad of 3- to 4-week-old ovariectomized athymic nude mice. Tumors formed spontaneously only in mice that were given injections of MCF-7/HRG cells. These tumors grew independently of the E2 stimulation and were resistant to antiestrogen treatments (Tam and ICI 172, 852) (Fig. 1, right and Table 1). In contrast, when mice were inoculated under the same conditions with MCF-7/V or the parental MCF-7 cells, the tumors appeared exclusively in the presence of E2 supplementation and were reduced significantly in the presence of antiestrogens (Fig. 1, left and Table 1). Interestingly, the MCF-7/HRG tumors generated in the presence of Tam were slightly larger. The data demonstrate that transfection of MCF-7 cells with HRG promotes tumor growth *in vivo* in the absence of E2 stimulation. These findings support the *in vitro* data from our laboratory, where HRG-transfected cells had a growth advantage when grown in E2-depleted medium (26). These results are analogous to the human disease, where, in the progression process, breast carcinomas acquire antiestrogen resistance, and thus, the results provide us with an extremely useful model for the study of breast cancer progression.

#### Anatomical Pathology of the HRG-Derived Tumors in Ovariectomized Athymic Nude Mice

We performed both macroscopic and microscopic analysis of all derived tumors. The majority of the tumors generated by the MCF-7/HRG cells presented as firm, poorly defined masses, with extensive, centrally located areas of necrosis. The lesions measured about 2 cm in greatest dimension. In several cases, in the MCF-7/HRG-derived tumors, we observed rib involvement and fixation to the underlying soft tissue, as well as erosion of the overlying skin. In the same group, enlargement of axillary lymph nodes was detected in many cases, whereas no macroscopic metastatic foci to visceral organs were noted. The MCF-7/HRG cell group exhibited tightly cohesive areas of large, pleomorphic cells with irregular nuclei, and numerous mitotic figures characterized the tumors. Multinucleated cells were observed. Irregular gland formation and well-formed central lumina as well as neovascular formation, and vast areas of necrotic tissue were observed. Infiltration of the adipose tissue and muscle, perineural invasion, and ribs involvement were seen. Neoplastic emboli were observed in both lymphatic vessels and blood. In a few cases, lymphoplasmacytic infiltrate was present at the periphery of the tumoral masses. Tumors derived from MCF-7/HRG showed a mixture of solid, trabecular, and tubular patterns. Irregular gland formation and occasional well-formed lumen were present. These features, as well as the heterogeneity and variety of histological patterns, resemble what is observed in the human mammary infiltrating ductal carcinoma termed “no special type A” (Fig. 2A).
MCF-7/HRG-Derived Tumors Metastasize to the Axillary Lymph Node

In the majority of the cases, the enlargement of the lymph nodes was attributable to the inflammation. However, in many mice, tumor deposits were seen in lymph node subcapsular sinuses and adjacent nodal tissue (Fig. 2B). Lymph node metastases were observed only in nude mice inoculated with MCF-7/HRG cells (Fig. 2C). These results demonstrate that HRG expression induces an E2-independent, antiestrogen resistance, and aggressive metastatic phenotype. This observation is unique, because to the best of our knowledge, no other known growth factor promotes metastasis of the MCF-7 cells, even in the presence of E2. These data support the concept that HRG is able to mediate tumor promotion and furthermore is an important factor in the progression of breast carcinomas.

HRG Is Highly Expressed in All the MCF-7/HRG-Derived Tumors

To clearly demonstrate that the tumors developed from MCF-7/HRG cells indeed expressed HRG, we performed in situ hybridization using a 22-bp oligonucleotide corresponding to the antisense sequence of HRG. Control sense probe was used as a negative control. HRG expression was undetectable in the MCF-7/V tumors when using the antisense HRG oligonucleotide (Fig. 3A). In contrast, the level of HRG expression in the tumors developed from MCF-7/HRG cells was extremely high when using the antisense oligonucleotide (Fig. 3B). No expression of HRG was seen in either tumor when using the sense oligonucleotide (Fig. 3C and D). Therefore, the expression of HRG is maintained in vivo and that the phenotypic changes are most probably mediated through HRG action. Interestingly, the level of HRG expression in the MCF-7/HRG tumors was comparable to the levels of HRG in tumors derived from MDA-MB-231 cells that naturally express HRG (data not shown).

MCF-7/HRG Cells Induce in Vivo Preneoplastic Transformation of the Adjacent Mammary Gland

A fascinating in vivo finding was the presence of atypical mouse glands, presenting features that appear reminiscent of ductal carcinoma in situ (Fig. 4A, left and right panels). The appearance of the preneoplastic-transformed glands is completely distinct from the normal appearance of the glands as a thin one-cell layer of epithelial cells. Microscopic examination of the mammary gland led us to consider that the malignant appearance is more likely attributable to the action of a paracrine factor and not to direct tumoral invasion. Because the preneoplastic transformation occurred only in the ovariectomized mice, inoculated with the MCF-7/HRG cells, we concluded that the transformation of these tissues could only be due to the secreted HRG from the MCF-7/HRG tumors. To rule out that the preneoplastic lesions were derived from the implanted MCF-7/HRG, we prepared tissue sections and stained them with two human specific antibodies, an anti-epithelial mammary antigen (EMA) (Fig. 4B, panels i and ii) and an anti-keratin (KER) (Fig. 4B, panels iii and iv). None of
these antibodies recognizes tissue of mouse origin. As can be seen, the neoplastic transformation of the mammary gland was of mouse origin. These findings are of extreme significance as they are the first to report that an HRG-derived tumor appears to promote the preneoplastic transformation of the adjacent tissues in a paracrine fashion \textit{in vivo}.

**VEGF Expression Is Up-Regulated in the MCF-7/HRG-Derived Tumors**

Because the tumors demonstrated a clear macroscopic increase in neovascularization, the tumors were stained for a known angiogenic factor, VEGF. The MCF-7/HRG tumors showed a high level of VEGF expression as shown by immunohistochemistry staining with anti-VEGF antibody (Fig. 5A).

These results were further confirmed by an ELISA assay, in which VEGF concentrations were measured in the conditioned media (CM) collected from MCF-7/HRG and MCF-7/V cells. A 3- to 8-fold increase in VEGF expression was observed in the CM from HRG-transfected cells (Fig. 5B). The levels of VEGF secreted by the HRG-derived MCF-7 clones directly correlated with the levels of HRG expression (data not shown).

**MAPK Is Activated in the MCF-7/HRG Cells**

To understand the mechanism by which HRG promotes this aggressive \textit{in vivo} phenotype, we examined the signaling pathways that could lead to such events. Previous studies \textit{in vitro} support the notion that transcriptional up-regulation of
VEGF can be mediated by MAPK (27, 28). In addition, the ras signaling pathway, and more precisely p42/p44 MAPK, are involved in the invasion process, and in the up-regulation of metalloproteinases as well as in the signals mediated by erbB2 (29). MCF-7 and MCF-7/HRG cells were therefore assayed for MAPK activation and expression. The constitutive levels of phosphorylated MAPK in MCF-7/HRG cells were at least 3-fold higher than in the parental MCF-7 cells (Fig. 6B), when the levels of phosphorylation were normalized to total MAPK protein that were loaded (Fig. 6C). This increase was comparable to that obtained when the parental MCF-7 cells were exogenously treated with HRG (Fig. 6B). Because MAPK is a known downstream effector of the erbB receptors, we further investigated if an increase in the phosphorylation status of these receptors was observed in the MCF-7/HRG cells. An increase in constitutive erbB receptor activation was observed in the MCF-7/HRG cells (Fig. 6A and Ref. 10), implying that the phosphorylation of MAPK can be mediated via the autocrine stimulation of erbB without overexpression of erbB2, as seen here in MCF-7 cells.

MCF-7/HRG Cells Secrete High Levels of Activated MMP-9

Because HRG induced an aggressive/metastatic behavior of MCF-7 cells, and MMP-9 is implicated in breast cancer progression, we speculated that modulation of MMP-9 activity might be the mechanism through which HRG induces the aggressive phenotype of MCF-7 cells. Thus, CM derived from MCF-7/HRG cells was analyzed by reverse zymography for MMP-9 activity. These assays demonstrated that the MCF-7/HRG cells produce significantly higher levels of MMP-9 activity (10-fold) as compared with control MCF-7/V cells (Fig. 7). Furthermore, the MMP-9 inhibitors R94138 (kindly provided by Dr. Kurakata at Sankyo, Japan) and GM6001 (Chemicon) blocked the ability of the MCF-7/HRG cells to invade through Matrigel using the Boyden Chamber assay (data not shown). We conclude that HRG expression is associated with increased MMP-9 activity and correlates with the metastatic potential of a breast cancer cell. Further studies are currently being conducted to confirm this hypothesis.

Discussion

Our data demonstrate that HRG expression is sufficient for the progression of MCF-7 cells to an E2-independent and antiestrogen-resistant state, supporting our previous results where HRG not only blocked E2 action but also ER function (26, 30). It is known that many breast cancer patients develop ER+ tumors that initially respond to antiestrogen treatment, but during the course of the treatment, the tumors acquire antiestrogen resistance. Although these tumors still express some levels of ER, they do not respond to antiestrogen treatments. Our in vivo model precisely mimics this scenario and shows that the MCF-7/HRG-derived tumors display a very similar process. To the best of our knowledge, this is the first report to demonstrate that the up-regulation of a single growth factor, such as HRG, is sufficient to promote such significant and critical effects in vivo. Furthermore, these effects are not accompanied by erbB2 overexpression. These findings are also of clinical importance, because they provide new tools to predict the outcome of breast cancer by evaluating the expression of HRG in the tumors, and also to predict which of the breast cancer patients may benefit from adjuvant therapy.

Our results show not only that the MCF-7/HRG became E2 independent but that they also became metastatic in vivo. The metastatic potential of cancer cells is influenced by a variety of signals such as growth factors, stroma cells, and ECM.
receptors. HRG increases the invasive phenotype of MCF-7 cells in vitro, as shown by migration in Matrigel, and also in a recently developed three-dimensional model (10, 31). However, this is the first report of increased metastatic ability of MCF-7 cells independent of any additional stimuli in vivo. These results show that HRG not only supports the formation of localized tumors in the absence of E2, but also mediates the development of metastases, which is the greatest clinical problem in the treatment of breast cancer. Transforming growth factor α, which is another mitogen for MCF-7 cells, failed to increase the ability of MCF-7 cells to form tumors in athymic-ovariectomized mice in the absence of E2 (32, 33). Other studies substantiate the hypothesis; for example, those from Weinstein and Leder (33), which demonstrated that preneoplastic tumors arise in HRG transgenic mice, where HRG was expressed under the control of the murine mammary tumor virus (MMTV) promoter. Even further, this group showed that HRG is involved in the development of the mammary gland, possibly through the regulation of the apoptotic process, a pathway that is involved in proper development of most organs. These results and ours show that the deregulation of HRG expression can play a dual role both in the promotion and the transformation of the mammary gland, and also in tumor progression and metastases. These reports strengthen the role of HRG not only in the proliferation of the cells but also in the metastatic process. The fact that the surrounding, mouse-derived, tissue was preneoplastic is astonishing. Here we show that the paracrine action of HRG can cause changes in the

![FIGURE 4. Preneoplastic transformation of the mammary gland in vivo. A. Tissue sections from mouse mammary glands adjacent to tumor areas derived from MCF-7/HRG cells. Sections were stained with H&E. Magnification, ×100. B. The transformed mammary gland is of mouse origin. The sections containing the transformed mammary glands were independently stained for EMA (panels i and ii) and for KER (panels iii and iv). Specific staining was developed using alkaline phosphatase. All sections were counterstained with Mayer hematoxylin. Magnifications, ×100 (top panel) and ×200 (bottom panel).](image-url)
mammary gland, which lead to the preneoplastic transformation of the tissue. Further investigation is required to establish which of the mammary gland cell types are the targets of HRG action. This study therefore shows that the HRG growth factor is not only a progression but also a transforming factor, which transforms normal epithelium. Screening for the up-regulation of HRG in breast tumors together with the currently available markers may therefore provide a useful tool to predict the effects of adjuvant therapy. Our data offer critical support for the concept that HRG overexpression could single out patients who may have a recurrence of the disease, who are thus at high risk for metastases.

The implications of these findings from a clinical point of view are of great importance placing HRG as a critical factor not only in breast cancer progression but also as a possible diagnostic and prognostic marker for the aggressiveness of the tumors and the devastating effects that the progression may have in patients where up-regulation of HRG expression is observed. Some of the effects that are observed in vivo may be also mediated indirectly via the up-regulation of other genes in an autocrine/paracrine manner. It is possible that the paracrine effects seen in the mammary gland are an effect of other secreted factors regulated by HRG, such as the newly discovered angiogenic factor, Cyr61 (34, 27). We have shown

FIGURE 5. VEGF expression is up-regulated in the HRG-derived tumors. A. The sections were stained using an anti-VEGF antibody (panel i). Immunohistochemical staining of MCF-7/HRG-derived tumors from E2-treated animals. Panel ii, peptide blocking of the VEGF antibody in MCF-7/HRG-derived tumors. Panel iii, staining with VEGF antibody of the MCF-7/V-derived tumors. Panel iv, specific blocking of the anti-VEGF binding by a VEGF-derived peptide in MCF-7/V-derived tumors. B. VEGF expression is up-regulated in the MCF-7/HRG cells. MCF-7/V and MCF-7/HRG cells were starved of serum for 24 h; CM was collected and assayed as per the manufacturer’s instructions.
that Cyr61 expression is significantly up-regulated in the MCF-7/HRG-derived tumors (35), suggesting that some of the phenotypic changes can be mediated through this factor. This is an ongoing area of investigation in our laboratory.

One of the key factors in tumor growth and survival is the ability of the mass to form new vasculature. VEGF has been implicated in numerous studies as a critical factor that regulates the process of angiogenesis. VEGF can be unregulated in MCF-7 cells after exogenous treatment with HRG (17). In addition, there is significant evidence that HRG mediates neovascularization in chick embryo model systems and in transgenic mice, where HRG expression was driven by the MMTV promoter (16, 17). Our study is the first to demonstrate that HRG regulates neovascularization of human breast cancer tumors in vivo in an autocrine manner. Moreover, our study provides direct evidence that subsequent to the dysregulation of HRG expression, the up-regulation of angiogenic factors occurs, which may promote tumor progression and neovascularization in vivo.

Transformation through the erbB2 oncogene has been proposed to involve both ras-mediated and ras-independent pathways (36). Furthermore, we have shown that downstream of HRG, Akt kinase, but not MAPK can be inhibited by down-regulation of Grb2, a protein that is associated with many tyrosine kinase receptors, including erbB2 (37). A recent study has shown up-regulation of ras and MAPK in breast cancer tumor sections (38). This activation was achieved in vitro only after treatment of the cells with ligands to growth factor receptors (38). Our model provides critical and clear evidence for the activation of MAPK via autocrine stimulation of erbB by HRG, suggesting that in tumors where HRG expression is up-regulated, activation of MAPK can occur in the absence of other ligands. Our finding demonstrating that MAPK is activated in these cells provides a new insight into the molecular events that may occur in an in vivo situation where resistance to antiestrogens is mediated by HRG via MAPK.

Possible crosstalk between the E2 and the MAPK pathways is supported by studies in which ERα can be phosphorylated by MAPK. These studies, however, were performed in vitro and do not take into account the other stimuli, such as the ECM, growth factors, and cytokines, all of which may contribute to MAPK activation in vivo. These findings support the notion that the up-regulation of HRG expression is a crucial event in the tumor progression in vivo, and also indicate that our in vivo model follows the course of the human disease most closely, where ras and MAPK are activated in the absence of ras mutations, and correlate with an aggressive and metastatic phenotype (38). It was previously shown that metastatic potential induced by activated ras was correlated with MMP-9 release (39). MMP-9 expression was shown to be necessary for this process, because a ribozyme directed against MMP-9 completely abolished the ability of these cells to metastasize (40). The possible mechanism by which HRG induces this aggressive phenotype is mediated by the alteration of the actin cytoskeleton morphology, by increased cellular motility, and by the invasion into the fibronectin-gelatin cross-linked matrix (7). We have shown that MMP-9 and MAPK activities are up-regulated in the HRG-transfected cells. Therefore, we hypothesize that the phenotype may be mediated via the modulation of MAPK and MMP-9 activity.

**FIGURE 6.** MAPK and erbB phosphorylation are up-regulated in MCF-7/HRG cells. MCF-7/HRG cells (clones T6, T7, and T8), and MCF-7/V cells (5 × 10⁵) were serum starved overnight. MCF-7/V cells (5 × 10⁵), used as control, were treated with HRG1 for 15 min at 37°C, under serum-free conditions. A. erbB phosphorylation in MCF-7, MCF-7/V cells treated with HRG, and MCF-7/HRG-transfected clones T6, T7, and T8. B. Phospho-MAPK in the MCF-7 ± HRG treatment, MCF-7/V cells ± HRG treatment, and in the T6, T7, and T8 clones. C. Total MAPK protein in the cells.
Our results provide a unique and faithful in vivo model for the progression of breast cancer, as well as possible mechanisms for the acquired E2 independence and antiestrogen resistance that is an enormous difficulty in the treatment of breast cancer. This model places HRG as a key role-player in the process, and in contrast with other studies, our findings show that the autocrine/paracrine effect of HRG is sufficient to promote both the E2 independence of the human-derived cells in vivo, and in the transformation of the mammary gland of mouse origin.

Although similar studies have been done in HRG transgenic mice, these animals were not ovariectomized, and therefore the growth of the tumors was in the presence of E2. This is, to the best of our knowledge, the first study to demonstrate that HRG promotes the progression of breast cancer cells in vivo from an E2-dependent to an E2-independent phenotype and that this is associated with increased MAPK activation, which may lead to increased expression of several angiogenic factors. Ongoing studies in our laboratory are investigating the role of MAPK increased expression of several angiogenic factors. Ongoing studies are currently underway to address whether blocking the individual signaling pathways induced by HRG could halt tumor progression and metastasis in vivo.

Materials and Methods

Cell Culture

MCF-7 cells were purchased from the American Type Culture Collection and routinely cultured in phenol red-containing IMEM (Biofluids, Camarillo, CA), supplemented with 5% (v/v) fetal bovine serum and 2 mM L-glutamine, at 37°C in a humidified atmosphere with 5% CO2, unless otherwise specified. MCF-7 cells were transfected with HRG cDNA as previously described (26).

Tumorigenicity Assay

Stably transfected MCF-7/V and MCF-7/HRG cells (1 × 10^6) were inoculated into the mammary fat pads of 3- to 4-week-old female ovariectomized athymic Nu/Nu nude mice in the presence or absence of E2 pellets (0.25 mg). The slow-release pellets (90-day release) were implanted subcutaneously to the cervical scapular space. The sizes of tumors were measured twice a week. Tumors were harvested, fixed in formalin, and embedded in paraffin when tumors reached up to 1.5 cm in diameter. The obtained tissues were partly frozen and partly fixed in PBS containing 10% formaldehyde, paraffin embedded, and stained with H&E for routine examination and histopathological studies.

**Immunohistochemical Staining and ELISA**

Formalin-fixed, paraffin-embedded breast tumor sections were deparaffinized in xylene and hydrated in graded alcohol series. Slides were quenched for endogenous peroxidase activity in the presence of 0.3% H2O2 for 30 min and blocked with 10% (v/v) horse serum for 30 min. This was followed by incubation with an anti-epithelial mammary antigen antibody, or anti-keratin antibody, or an anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). To verify that VEGF staining was specific, a VEGF peptide was used to block any possible nonspecific staining. The sections were washed in PBS before the incubation with a biotinylated secondary antibody for 30 min. The sections were then incubated with avidin-biotin complex (VECTASTAIN Elite ABC reagent, Vector Laboratories, Burlingame, CA) for 30 min, and the reaction was developed in the presence of H2O2 and 3,3'-diaminobenzidine tetrahydrochloride (DAB). VEGF quantification was performed using CM derived from MCF-7/HRG cells and MCF-7/V cells using a specific anti-VEGF ELISA assay (R&D Systems, Inc., Minneapolis, MN). MCF-7/V and MCF-7/HRG cells were starved of serum for 24 h; CM was collected and assayed as per the manufacturer’s instructions.

*In Situ Hybridization*

MCF-7 cells and MCF-7/HRG-transfected cells were fixed and embedded in paraffin. Fixed and permeabilized cells were treated with proteinase K for 20 and 30 min. The slides were hybridized overnight at 55°C with 1 × 10^6 DPM of 35S-labeled antisense probe in a solution containing 50% formamide, 2 g of dextran sulfate, 50 mM Tris, 500 ml tRNA, and 400 ml Denhardt’s solution. The slides were washed, dipped in NTB2 emulsion, and stored in light-tight boxes. After suitable exposure times, the slides were developed and stained with H&E.
Western Blot Analysis

MCF-7/HRG cells (clones T6, T7, and T8) and MCF-7/V cells (5 x 10⁵) were plated in IMEM containing 5% fetal bovine serum, in six-well plates. After 24 h, cells were washed three times with serum-free media, and serum starved overnight. MCF-7 cells (5 x 10⁶), used as control, were treated with HRG/91 (Neomarkers, Fremont, CA) (30 ng/ml) for 15 min at 37°C, under serum-free conditions. All cells were lysed with lysis buffer, consisting of 50 mM HEPES, 1% Triton X-100, 0.15 M NaCl, 10% glycerol, 2 mM EDTA, and 50 μM ZnCl₂; protein quantification was performed using the BCA reagent (Pierce Chemical Co., Rockford, IL), and 50 μg of total protein of each sample was loaded onto a 12% SDS-PAGE. The proteins were transferred onto nitrocellulose membranes and blotted with anti-MAPK, anti-phospho-MAPK antibodies (Cell Signaling Technology, Beverly, MA), or anti-phosphotyrosine 4G10 clone (Upstate Biotechnology, Inc., Lake Placid, NY). The membranes were developed using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Zymography Analysis

Near-confluent breast cancer cells were cultured in serum-free media for 24 h and fed with serum-free media for another 48 h. The CM were collected and concentrated by centrifugation (Millipore, Bedford, MA) at 4°C. Protein concentration was determined and equal amounts of protein were separated by 10% gelatin/10% acrylamide SDS-PAGE. Gels were washed in renaturing buffer [50 mM Tris-HCl (pH 7.6) and 2.5% Triton X-100] for 30 min, and in TNCB enzyme buffer [50 mM Tris-HCl (pH 7.6), 0.2 M NaCl, 5 mM CaCl₂, and 0.02% Brij-35] for 30 min. Gels were then incubated in TNCB enzyme buffer for at least 20 h at 37°C and stained with 0.5% Coomassie brilliant blue R250.

Acknowledgments

We thank Dr. N. Smorodinsky for scientific discussion and Kevin Peet for editorial and continuous support. We also thank Jessica Castaneda for technical assistance during the course of this work.

References


Molecular Cancer Research

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Mol Cancer Res 2003;1:165-175.

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