Development of an Autocrine Neuregulin Signaling Loop with Malignant Transformation of Human Breast Epithelial Cells

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ABSTRACT

Neuregulin (NRG) is a heparin-binding factor that activates members of the epidermal growth factor family of tyrosine kinase receptors including erbB2 that is overexpressed in more aggressive types of breast cancer. The exact role that NRG plays in breast cancer is complicated by the fact that NRG has been shown to have both proliferative and antiproliferative effects, depending on the breast cancer cell line used. Using an isogenic series of breast epithelial cell lines (MCF10A) ranging from benign to malignant, we found that the actions of NRG changed from antiproliferative to proliferative as the cells progress to cancer. This correlated with a progressive inability of NRG to down-regulate a group of proliferation genes identified previously using cDNA microarrays. As the cells progress to malignancy, they expressed higher levels of erbB2 and lower levels of erbB3 and secreted high levels of NRG into the culture media, resulting in high basal levels of erbB receptors phosphorylation. Disruption of this autocrine signaling loop by blocking ligand-induced receptor activation inhibited cancer cell proliferation. These results demonstrate that the transition of MCF10A cells from normal to premalignant to malignant correlates with the development of a constitutively active autocrine NRG signaling loop that promotes cell proliferation and suggest that disrupting this autocrine loop may provide an important therapeutic measure to control breast cancer cell growth.

INTRODUCTION

The family of growth and differentiation factors collectively called neuregulins (NRGs) was discovered when researchers tried to find ligands that activated the neu oncogene (erbB2) using breast epithelial cells (1, 2). NRG was initially named neu differentiation factor by one group because it was a 44-kDa glycoprotein that induced phenotypic differentiation of cultured mammary tumor cells to growth-arrested, milk-producing cells (3). Another group named it heregulin, for a factor purified from the conditioned media of a human breast tumor cell line that promoted cell growth through erbB2 receptor activation (4). Shortly thereafter, it was discovered that NRG also plays essential roles in the normal growth and development of the nervous system and heart and promotes breast development, pregnancy, and lactation (1, 4–8). In vivo, overexpression of NRG induces breast tumor formation in transgenic mice (9) and promotes the formation of Schwann cell tumors when overexpressed in peripheral nerve (10).

Binding of NRG to homo- and heterodimers of erbB2, erbB3, and erbB4 receptors results in rapid receptor phosphorylation, initiating a signaling cascade that translates this initial binding event into specific gene expression changes. Although NRG was originally isolated as the putative ligand for erbB2, NRG does not bind erbB2 and only transactivates erbB2 through direct binding to the other erbB receptor family members erbB3 and erbB4 that heterodimerize with erbB2 (9, 11–14). Evidence suggests that erbB2 overexpression occurs in nearly 30% of human breast tumors and is associated with a poor prognosis (15–17). The exact mechanism of how erbB2 overexpression contributes to the malignancy of breast epithelial cells is unclear. Nonetheless, a number of successful treatment strategies have been used to target the erbB2 receptor, including, a humanized monoclonal antibody called trastuzumab (Herceptin; refs. 18–21).

Depending on the breast cell line used, both proliferation and differentiation effects can be produced by NRG (1, 22, 23). This creates a conundrum of how activation of the same receptors results in different, cell type-specific biological effects (4, 24). For example, sustained activation of the mitogen-activated protein/extracellular signal-regulated kinase pathway is both essential and sufficient for NRG-induced growth arrest and differentiation in AU565, MDA-MB-453, and SKBR3 cells, whereas NRG promotes cell proliferation and tumorigenesis in T47D and HC11 cells (4, 24–26). Other reports have shown that cells that express normal levels of erbB receptors are growth stimulated by NRG, whereas SKBR3 and other erbB2-overexpressing breast tumor cells are growth inhibited (27). However, this was not the case in another study (22), in which NRG had proliferation effects both in vitro and in vivo in all cell lines studied, particularly in those cell lines that overexpressed erbB2.

To complicate things further, many breast tumor cells synthesize NRG themselves. In fact, NRG was originally purified from MDA-MB-231 breast epithelial cell media (3), and endogenous NRG has been shown to be involved in breast cancer tumor progression (17, 28, 29). Furthermore, the expression of endogenous NRG in breast cancer cell lines correlates with a more malignant phenotype, and genetically engineered cells that constitutively express NRG have phenotypic changes that lead to enhanced aggressiveness and invasiveness (17). In fact, endogenous NRG production has been postulated to produce an autocrine signaling loop in ovarian and colon cancers as well as in transformed Schwann cells that could play critical roles in cancer formation (30–32).

The plethora of different breast cell lines in existence has greatly contributed to the confusing aspects of the actions of NRG. One way to get around this is to examine a series of cell lines derived from a single, clonal cell line. The human MCF10 series was developed for just this reason by spontaneous immortalization of normal breast epithelial cells from a patient with fibrocystic disease (33). The MCF10 cell series is a well-developed model system that consists of a well-differentiated line called MCF10A, a premalignant, ras-transformed line called MCF10AT, and a fully malignant subclone from this line called MCF10CA1 (33). MCF10A cells are unable to form lesions in nude mice; all epithelial cells disappear within 4 weeks after xenografting (34). The MCF10AT cells can generate benign ductal structures in mice with a potential for malignant tumor progression that occurs in approximately 25% of mice during their life span. The MCF10CA1 cells produce highly invasive cancers immediately after transplantation into nude mice (35). Earlier studies have shown that NRGs can substitute for epidermal growth factor (EGF) and insulin-like growth factor to increase the growth rate and promote anchorage-independent growth of this cell line under serum-free, growth factor-deficient conditions (36, 37). When transformed with either H-ras or erbB2, increased expression of NRG forms was found,
leading to the suggestion that these transformations induce autocrine NRG signaling (37). The MCF10 model thus covers the complete spectrum of tumor progression from relatively normal breast epithelial cells to breast cancer cells capable of metastasis and provides a unique tool to investigate the role of NRG in cell growth properties.

In this study, we used the MCF10 series to investigate how NRG affects breast epithelial cell growth. Using cDNA microarray analysis, we found recently that NRG reduces the growth rate of MCF10A/AT cells in correlation with a marked down-regulation of a group of NRG response genes, many of which have been shown to be up-regulated during cell proliferation (38). We now show that as cells in the MCF10 series become progressively malignant, exogenous NRG treatment of these cells results in a change from antiproliferative to proliferative effects with the concomitant failure of NRG to down-regulate these genes. Using several different NRG and NRG signaling antagonists, we show that malignant progression is associated with the development of a proliferative autocrine NRG signaling loop, associated with a reduction of erbB3 expression and the overexpression of both erbB2 and NRG.

MATERIALS AND METHODS

Reagents and Cell Lines. Insulin and hydrocortisone were purchased from Sigma (St. Louis, MO); EGF was from Upstate Biotechnology (Lake Placid, NY); cholera toxin and AG1478 were purchased from Calbiochem (La Jolla, CA). All of the other media, buffer, and ingredients for cell culture were purchased from Invitrogen Life Sciences (Carlsbad, CA). NRG β1 recombinant protein IG-EGF form, which corresponds to amino acids 14–276, was provided by Amgen (Thousand Oaks, CA). Trastuzumab was a generous gift from Dr. Wei-Zen Wei (Barbara Kammans Institute, Wayne State University, Detroit, MI). Phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology; erbB2 and erbB3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat antianti serum and goat antirabbit antibodies were from Chemicon (Temecula, CA). Normal breast epithelial MCF10A, prema- lignant MCF10AT, and malignant MCF10CA1 cells were provided by the Barbara Ann Karmanos Cancer Institute Core Cell Facility (Wayne State University, Detroit, MI). The NRG antagonist IgB4 stably transfected HEK293 cells were a gift from Dr. Gabriel Corfas (Harvard University, Cambridge, MA), and the plasmid was originally from Dr. Yosef Yarden (Weizmann Institute, Rehovot, Israel).

Cell Cultures. The MCF10A and MCF10AT cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM):Ham’s F-12 supplemented with 5% horse serum, 10 mmol/L HEPES buffer, 10 ng/mL insulin, 20 ng/mL EGF, 100 ng/mL cholera toxin, and 0.5 mg/mL hydrocortisone at 37°C in a 5% CO2 incubator. The MCF10CA1 cells were cultured in DMEM:Ham’s F-12 with 5% horse serum in a 5% CO2 incubator. Cells were fed twice a week and passaged on a weekly basis. The L6 cells were cultured in DMEM supplemented with 10% fetal calf serum, 1 mmol/L L-glutamine, and 1,000 units/mL penicillin/streptomyacin at 37°C in a 10% CO2 incubator as described previously (39). The NRG antagonist IgB4 stably transfected HEK293 cells were cultured in DMEM with 10% fetal calf serum, 1 mmol/L L-glutamine, 2 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acid, 1,000 units/mL penicillin/streptomycin, and selective antibiotic Geneticin (200 μg/mL) at 37°C in a 10% CO2 incubator.

Cell Proliferation Assays. All three breast epithelial cell lines were plated at 5,000 cells per well in 48-well plates in regular MCF10A/AT or MCF10CA1 media. After incubation for 3 days, 1 mmol/L NRG in MCF10A/AT media or MCF10A/AT media alone was added to the cells for another 24 hours, followed by washing them with culture media and then counting cell numbers using a hemocytometer on days 4, 5, 6, 7, and 8.

RNA Preparation and Northern Blotting. MCF10A, MCF10AT, and MCF10CA1 cells were cultured in 25-cm flasks to approximately 60% conflu- ence. The cells were treated with 1 mmol/L NRG in MCF10A/AT media for 24 hours. The cells were then washed with PBS once and harvested. Total RNA was extracted from cells using Ultraspec (Biotec Laboratories, Inc., Houston, TX), “cleaned up” by RNeasy purification kit (Qiagen, Valencia, CA), and subsequently quantified using a fluorescent dye binding method called Ri-

bogreen (Molecular Probes, Eugene, OR). Northern blots were performed as described previously (40) on RNA extracted from the three breast epithelial cell lines after NRG treatment in MCF10A/AT media versus control MCF10A/AT media. Probes were generated by polymerase chain reaction and then prepared by random priming to full-length cDNA clones from the clones provided by Alphagene Inc. (Woburn, MA) as described elsewhere (38). The membranes were reprobed with a 3P-labeled glyceraldehyde-3-phosphate dehydrogenase probe for normalization.

Western Blots and L6 Muscle Assays. P185 receptor phosphorylation of the three cell lines was measured by phosphotyrosine Western blots after NRG treatment for 30 minutes on 3-day-old cultures as described previously (40). In experiments to block NRG-induced receptor activation, 1 nmol/L NRG with or without blocking reagents (IgB4, 10 μmol/L AG1478, or 100 μg/mL trastuzu-mab) was applied to 3-day-old MCF10CA1 cells for 30 minutes at 37°C in a 5%CO2 incubator. The medium was discarded, and phosphotyrosine Western blots were performed as described above. The membranes were stripped and reprobed with polyclonal rabbit erbB2 for the detection of overall erbB2 protein that was used for quantitation.

Concentrated culture media to assay for NRG from each cell line were prepared on 3-day-old cultures that had been placed in OptiMEM (Invitrogen) for an additional 2-day period. The conditioned media were concentrated using a Centricon device (Fisher, Hanover Park, IL) at 4°C. At the same time, cell numbers of the three cell lines were counted and used to normalize the amount of conditioned media per cell added to the L6 bioassay for quantifying the amount of NRG released into the media.

Reverse Transcription-Polymerase Chain Reaction. One microgram of total RNA isolated from either MCF10A, MCF10C0, or MCF10CA1 cells was used for reverse transcription-polymerase chain reaction (RT-PCR). The RT- PCRs were performed using the Superscript II RT-PCR system (Invitrogen) with 10 μL working primers that correspond to the heparin binding domain of human NRG β1 form: forward primer, 5-CAGGATCCAAAAGGAGGCGGAGGGTCCTC3; and reverse primer, 5-GGCTGATCTAATTTGCTATCACCTTGTC3. The cDNAs were resolved on 1% agarose gels and photo- graphed using Polaroid GelCam with Polaroid 667 film (VWR, Chicago, IL).

IgB4, AG1478, and Trastuzumab Treatments. NRG antagonist IgB4 stably transfected HEK293 cells were cultured to 80% confluence before OptiMEM I was applied to the cells. After 2 days, the OptiMEM I-conditioned media were concentrated by Centricron at 4°C. Forty microliters of IgB4-conditioned media or 10 μmol/L tyrosine kinase inhibitor AG1478 were combined with 150 μL of 1 nmol/L NRG in MCF10A/AT media for 15 minutes at room temperature before being added to MCF10CA1 cells for a 30-minute treatment. For the trastuzumab experiment, the MCF10CA1 cells were treated with 100 μg/mL trastuzumab in MCF10CA1 media for 24 hours before the cells were treated with 1 nmol/L NRG in the presence of 100 μg/mL trastuzumab in MCF10CA1/AT media for 30 minutes. The amount of each antagonist was determined empirically, based on the ability of the antagonist to block NRG-induced activation of L6 cells.

RESULTS

Neuregulin Changes from an Antiproliferative to a Proliferative Factor in the MCF10 Series and Loses the Ability to Down-Regulate Cell Proliferation Genes. We investigated how NRG af- fects the proliferation rate of the MCF10 series of cell lines (MCF10A, MCF10AT, and MCF10CA1) grown in serum-containing media (Fig. 1). Three days after plating, 1 nmol/L NRG treatment for 24 hours produced a sustained reduction in the growth rate of the MCF10A cells. In contrast, NRG treatment increased the initial growth rate of the more malignant MCF10CA1 cells. NRG treatment of the MCF10AT cells resulted in only a small, initial reduction in growth rate, an effect that fell in between that of NRG on MCF10A and MCF10CA1 cells. These results demonstrate a gradual transition in the effects of NRG on the growth of MCF10 cell lines that switches from an antiproliferative effect to a proliferative effect as the cells become more malignant.

Cell growth is a complicated process that involves many genes. We have recently used cDNA microarrays with Northern blot confirma-
tion to identify a group of NRG response genes that were coordinately down-regulated in the first 24 hours of NRG treatment of MCF10AT cells (38). Many of these genes could be considered “proliferation” genes, and they include several oncogenes, cell cycle control genes, and cell proliferation genes. We chose a representative group of eight of these NRG response genes that changed in response to NRG treatment in each of the three MCF10 cell lines (Fig. 2). Northern blots were performed on total RNA isolated from each cell line treated with or without 1 nmol/L NRG for 24 hours. Without NRG treatment, the most malignant cell line, MCF10CA1, expressed these genes at the highest baseline levels compared with the MCF10A cells, whereas the MCF10AT cell line expressed them at the lowest levels, thus requiring longer exposures (shown on the right of Fig. 2A). NRG treatment produced a rapid down-regulation of all eight of these genes to varying degrees in both the MCF10A and MCF10AT cells; however, as shown in Fig. 2B, the effect was significantly greater for the MCF10A cells than for the MCF10AT cells. This is consistent with the effects of NRG on cell proliferation described above, where NRG had only transient effects on the MCF10AT cells. In contrast, there was very little down-regulation of just a few of these genes in the MCF10CA1 cells that increased their proliferation rate with NRG treatment. Thus, proliferation genes are both basally up-regulated and no longer effectively down-regulated by NRG in the more malignant MCF10CA1 cells, despite growing at a similar rate as the other cell lines (see Fig. 1). These results raise an important mechanistic question with regard to how signaling by the same growth factor leads to such different effects on cell proliferation in the MCF10 cell line series.

The Neuregulin Proliferation Response Correlates with Increased Expression of erbB2, Decreased Expression of erbB3, and Increased Secretion of Endogenous Neuregulin. One possibility is that differential erbB receptor expression and/or activation may contribute to the different responses to NRG in the MCF10 cell line series. In fact, MCF10A cells that overexpress erbB2 are more sensitive to the mitogenic effects of NRG (36). We therefore measured the degree of NRG-induced receptor tyrosine phosphorylation (p185) using a phosphotyrosine antibody and the relative abundance of erbB2 and erbB3 that can form active receptor heterodimers in MCF10A, MCF10AT, and MCF10CA1 cells (Fig. 3). Because p185 can be composed of multiple erbB receptors, we reprobed the phosphotyrosine Western blot in Fig. 3A with specific antibodies to erbB2 (Fig. 3B) and erbB3 (Fig. 3C). This demonstrated that the top band (p185)

Fig. 1. Differential effects of NRG on cell proliferation of breast epithelial cell lines MCF10A, MCF10AT, and MCF10CA1. Breast epithelial MCF10A, MCF10AT, and MCF10CA1 cells were plated at 5,000 cells per well in 48-well plates for 3 days. The cells were then treated with (▲) or without (○) 1 nmol/L NRG in MCF10A/AT media for 24 hours (arrow) before the cell numbers were counted on days 4, 5, 6, 7, and 8.

Fig. 2. NRG treatment differentially regulated expression levels of eight proliferation genes in the three cell lines. A. Northern blots were performed using total RNA isolated from each cell line treated with or without 1 nmol/L NRG for 24 hours. The eight genes, which included heat shock genes (M22832, L15189, M94859, and NM_006597), an oncogene (M19722), a cell cycle control gene (U47413), and genes involved in translation and metabolism (L41490 and Y00711), were analyzed for the three cell lines. Without NRG treatment, MCF10CA1 cells expressed much higher basal levels of these genes compared with MCF10A cells, whereas premalignant MCF10AT cells expressed far lower levels, thus requiring longer exposures, shown on the right of Fig. 2A. NRG treatment produced a rapid down-regulation of all eight of these genes to varying degrees in both the MCF10A and MCF10AT cells; however, as shown in Fig. 2B, the effect was significantly greater for the MCF10A cells than for the MCF10AT cells. This is consistent with the effects of NRG on cell proliferation described above, where NRG had only transient effects on the MCF10AT cells. In contrast, there was very little down-regulation of just a few of these genes in the MCF10CA1 cells that increased their proliferation rate with NRG treatment. Thus, proliferation genes are both basally up-regulated and no longer effectively down-regulated by NRG in the more malignant MCF10CA1 cells, despite growing at a similar rate as the other cell
in both the MCF10AT (6-fold) cells relative to the MCF10A cells. Both of these fold changes had statistically significant increases in erbB2 expression in MCF10AT (2-fold) and MCF10CA1 malignant MCF10CA1 cells (Fig. 3). At the same time erbB2 expression increased, erbB3 expression decreased in the MCF10AT cells. Both of these fold changes had P values of <0.001 using two-tailed Student’s t test (**). C. Reprobing the same blot with erbB3 antibodies demonstrated that predominantly the top band but also a smaller proportion of the bottom band contained erbB3. In contrast to erbB2, erbB3 expression decreased by almost 2-fold in both the MCF10AT (*, P < 0.01) and MCF10CA1 cells (**, P < 0.005).

The presence of high basal levels of p185 receptor phosphorylation in the absence of exogenous NRG in the malignant cell lines raises the possibility that these cells secrete NRG, which, in turn, activates the erbB2 and erbB3 receptors in an autocrine pathway. To test for the presence of NRG, we performed RT-PCR on MCF10A, MCF10AT, and MCF10CA1 cells using a specific primer pair to the heparin-binding domain of human NRG (Fig. 4A). This domain is expressed in all soluble forms of NRG (7). Although the results are not quantitative, they suggest that increasing levels of NRG are expressed as the breast epithelial cells become more malignant cancer cells. More directly relevant to our hypothesis, we measured the amount of NRG activity released into the conditioned media from equivalent cell numbers of each of the three cell lines. This was done using the L6 muscle cell line as a sensitive means to measure soluble NRG activity through p185 receptor phosphorylation (41). Fig. 4B shows that the amount of NRG activity in conditioned media from each of the three cell lines increased significantly as the cells became more malignant. This increase paralleled their degree of basal p185 phosphorylation, suggesting that endogenous NRG production is responsible for high basal levels of erbB receptor phosphorylation in the more malignant cells.

Existence of an Autocrine Loop Can Be Revealed by Neuregulin Antagonists. The results thus far raise the possibility that as the MCF10A cells become more malignant, they develop an autocrine NRG signaling loop that promotes cell proliferation. We therefore postulated that if we could block this autocrine loop in the highly malignant...
MCF10CA1 cells, we would be able to reduce their proliferation rate. We blocked NRG signaling using three complimentary approaches: (a) by blocking endogenous NRG from activating cell surface erbB receptors using a soluble erbB4 receptor antagonist called IgB4 (this consists of the extracellular domain of erbB4 fused to an immunoglobulin Fc domain to produce an antagonist that works by competing with cell surface receptors for soluble NRG released into the culture media; ref. 42); (b) by blocking erbB receptor signaling pharmacologically using an erbB receptor-specific tyrosine kinase inhibitor called AG1478 (43); and (c) by down-regulating erbB2 receptors with a specific monoclonal antibody called trastuzumab.

In MCF10CA1 cells, IgB4 effectively blocked both the high basal levels and exogenous NRG-induced p185 receptor phosphorylation (Fig. 5A and B). IgB4 not only blocked MCF10CA1 cell proliferation induced by exogenous NRG treatment but also substantially reduced the growth rate of these cells without exogenous NRG treatment (Fig. 5C). This suggests that endogenous NRG production is not only responsible for activating basal levels of erbB receptor phosphorylation but directly induces MCF10CA1 cell proliferation. The specific erbB receptor tyrosine kinase inhibitor AG1478 was able to block NRG-induced erbB signaling. AG1478 reduced both p185 (erbB2/3, top band in Fig. 3) and EGF receptor phosphorylation (bottom band of Fig. 3) in a dose-dependent manner (Fig. 6A and B). Similar to the IgB4, at an effective concentration of 10 μmol/L, AG1478 reduced the growth rate of the MCF10CA1 cells, both with and without exogenous NRG treatment (Fig. 6C). Finally, when cells were pretreated with trastuzumab, the basal levels of erbB receptor phosphorylation were not affected in MCF10CA1 cells. However, the response to exogenous NRG was reduced (Fig. 7A and B). Consistently, whereas trastuzumab reduced cell proliferation in response to exogenous NRG, it had no effects on cell proliferation in the absence of exogenous NRG (Fig. 7C).

Taken together, these results suggest that whereas the most effective way to block both NRG signaling and growth promotion is by disrupting an autocrine loop by reducing extracellular NRG with a soluble antagonist, blocking downstream erbB receptor phosphorylation or cell surface expression of erbB2 is also effective in reducing MCF10CA1 cell proliferation.

**DISCUSSION**

The work presented here shows that progressive malignant transformation of the MCF10 cell line is associated with a loss of the normal antiproliferative effects of NRG and the development of an autocrine NRG signaling loop that stimulates cell proliferation. The MCF10 cell series covers the whole spectrum of tumorigenesis from the fairly normal MCF10A cells to the premalignant MCF10AT cells to the highly malignant MCF10CA1 cells in a single, isogenic series. Effects of exogenous NRG on this series of cell lines “switched” from antiproliferative to proliferative as the cells changed from normal to malignant. This was associated with a relative increase in erbB2 and a decrease in erbB3 expression. Because erbB2 cannot bind NRG, and erbB3 has an inactive tyrosine kinase domain, the change in the proportion of erbB2/erbB3 could significantly alter NRG binding to erbB3 and signaling through erbB2. Whereas the mechanism for this change in NRG responsiveness may stem from changes in erbB receptor subtype expression, it may also have resulted from markedly higher levels of endogenous NRG secretion that produce a high basal level of erbB receptor activation through an autocrine signaling loop (Fig. 8). Consistently, disrupting this autocrine loop by several different approaches effectively reduced both sustained erbB receptor activation and the rate of cell growth.

Exactly what leads to this switch of NRG from an antiproliferative factor to a proliferative factor as these cells become more malignant is not clear. Because the MCF10AT cells were derived by ras transformation of MCF10A cells, activating the ras oncogene may be necessary but not sufficient for the preneoplastic phenotype (44, 45). An increase in the ratio of erbB2 to erbB3 could produce an altered receptor phosphorylation response, resulting in a change in signaling...
pathways that could promote cell proliferation and/or suppress the normal antiproliferative effects of NRG. Consistent with this later possibility, we found that NRG response genes increased normally during periods of cell proliferation are down-regulated by NRG in MCF10A and MCF10AT cells but are expressed at high basal levels and resistant to NRG in the more malignant MCF10CA1 cells. These genes were recently identified in a microarray screen of MCF10AT cells treated with NRG for 24 hours. They include heat shock genes, oncogenes, and cell cycle control genes often seen to be highly expressed in malignant, rapidly proliferating cells (38). Curiously, despite the basal up-regulation of these proliferation genes, the MCF10CA1 cells do not grow appreciably faster than the MCF10A and MCF10AT cells.

The difference in the effects of NRG on the growth of the malignant MCF10CA1 cells could also be due to a marked elevation in endogenous NRG secretion producing sustained, basal erbB receptor phosphorylation. Sustained receptor activation may be achieved in part from the accumulation of secreted NRG in the extracellular matrix. Using RT-PCR, we found that the MCF10 cell series express NRG isoforms containing a heparin-binding domain (7, 8). This heparin-binding domain has been shown to restrict NRG to the extracellular matrix of various cells during development and significantly potentiates its biological activities (39, 40, 46). Most previous studies have used recombinant NRG forms that lack this heparin-binding domain. In contrast, in our study, we used a naturally occurring form of NRG that contains the heparin-binding domain and thus would be expected to produce more sustained signaling responses through matrix interactions. This may be why others have observed proliferative effects of NRG on MCF10A cells where we found clear antiproliferative effects (36, 37). Another important difference between our study and these earlier studies that were focused on growth factor dependence is that they used serum-free defined media, whereas we included serum as well as EGF insulin in our cultures to try to provide a more normal basal background on which to measure the growth effects of NRG.

Sustained signaling may be one way by that cells can decide to proliferate or differentiate, based on the length of time they are exposed to a given growth factor. We have shown previously in muscle cells that a minimum of 8 hours of constant receptor activation is required for NRG-induced expression of acetylcholine receptors (40). In other systems, sustained receptor activation utilizes different signaling pathways that lead to different biological effects than those produced by transient receptor activation (32, 47, 48). In the MCF10A

Fig. 6. The tyrosine kinase inhibitor AG1478 blocked both erbB receptor phosphorylation and proliferation of MCF10CA1 cells. A, MCF10CA1 cells were treated with and without 1 nmol/L NRG in combination with AG1478 for 30 minutes, followed by Western blot analysis of p185 receptor phosphorylation. The membrane was reprobed for erbB2 receptor to normalize for protein loading. This antagonist also blocked the lower, EGF receptor band. B, Quantitation of the p185/erbB2 levels shows that IgB4 inhibited p185 receptor phosphorylation induced by both endogenous and exogenous NRG. C, Cell proliferation assays were performed to examine the effects of AG1478 on MCF10CA1 growth rate with and without the addition of NRG. AG1478 blocked both normal and NRG-induced cell growth to a similar level.

Fig. 7. The erbB2-specific antibody trastuzumab reduced erbB receptor activation but had no effect on NRG-induced proliferation of MCF10CA1 cells. A, MCF10CA1 cells were pretreated with 100 μg/mL of the erbB2 receptor–specific antibody trastuzumab for 24 hours, followed by treatment with or without 1 nmol/L NRG for 30 minutes. P185 receptor phosphorylation and erbB2 receptor expression were examined by Western blot. B, Quantitation of p185/erbB2 levels showed that trastuzumab did not affect erbB receptor phosphorylation when applied to MCF10CA1 cells alone but reduced NRG-induced erbB receptor activation. C, MCF10CA1 cells pretreated with 100 μg/mL trastuzumab (filled symbols) for 24 hours followed by either no treatment or treatment with 1 nmol/L NRG showed that trastuzumab did not significantly reduce their growth rate in the absence of added NRG.
that a focused approach that specifically disrupts NRG signaling may be blocked by disrupting NRG signaling in the most malignant cell line.

Autocrine loops for NRG signaling have been described previously both in breast cancer cell lines (3, 17, 37) and in cancer cells from other epithelial tissues. For example, endogenous NRG production has been found in a majority of ovarian cancers and in GEO colon cancer cells with both inhibition of apoptosis and cell growth that can be blocked by NRG or erbB receptor antibodies (30, 31). The development of a proliferative NRG erbB receptor autocrine signaling loop in the MCF10 series suggests that agents that specifically disrupt this autocrine loop could be therapeutically effective. For example, a NRG antisense cDNA used to block endogenous NRG expression in MDA-MB-231 cells successfully suppressed the aggressive and invasive phenotype of these cancer cells (28). Similarly, specific receptor tyrosine kinase inhibitors such as emodin and emodin derivative DK-V-47 have been used (49). One difficulty with many of these reagents as well as the erbB-specific tyrosine kinase antagonist AG1478 used here is a lack of specificity for NRG ligands. AG1478 blocks tyrosine phosphorylation of not only the erbB2 and erbB3 receptors but also of the EGF receptor. Trastuzumab is a humanized monoclonal antibody that binds to erbB2 and is currently used clinically in patients who overexpress erbB2. Trastuzumab had minimal effects on blocking exogenous NRG-induced p185 receptor phosphorylation of MCF10CA1 cells even after 24 hours of pretreatment. It also had minimal effects on reducing proliferation. Another monoclonal antibody, 2C4, which works by blocking the association of erbB2 and erbB3, was found by others to inhibit ligand-activated erbB2 signaling and cell growth regardless of the expression level of erbB2 protein, but it had no growth inhibition without exogenous ligand stimulation (20).

In summary, our results suggest that sustained erbB receptor activation through the autocrine effects of NRG is a key promoter of cell proliferation and may be part of a developmental program in breast epithelial cells that produces a malignant phenotype where proliferation genes are chronically up-regulated. Our results therefore suggest that a focused approach that specifically disrupts NRG signaling may be needed to target cancer cells and interrupt NRG autocrine signaling.

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