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The Extracellular Region of Heregulin Is Sufficient to Promote Mammary Gland Proliferation and Tumorigenesis but not Apoptosis

Edward J. Weinstein and Philip Leder[1]

Department of Genetics, Harvard Medical School, and Howard Hughes Medical Institute, Boston, Massachusetts 02115

ABSTRACT

Heregulin (HRG) is a member of the neuregulin family of ligands that have been shown to interact with and activate erbB receptors. A transgenic mouse model in which full-length HRG is overexpressed has proven that this protein can induce carcinomas in the murine mammary gland. These tumors display a high level of apoptosis, which appears to be mediated by the cytoplasmic domain of HRG. Because both proliferation and apoptosis play a role in tumor formation, we wished to separately view those perturbations by removing the suspected apoptosis-inducing cytoplasmic domain of HRG. We thereby sought to determine whether overexpression of the extracellular region of HRG would be sufficient to induce mammary gland carcinomas. A HRG construct lacking the cytoplasmic domain was targeted to the mammary gland using the murine mammary tumor virus promoter. Multiple lines of transgenic mice carrying the transgene developed mammary gland tumors at approximately 15 months of age. These tumors did not display high levels of apoptosis as compared with tumors from murine mammary tumor virus/full-length HRG transgenic animals. In addition, virgin transgenic mice show a persistence of terminal end bud structures, which normally disappear at the onset of puberty in wild-type mice. To examine the signal transduction pathway activated by extracellular HRG in tumors, we investigated the phosphorylation status of the epidermal growth factor receptor family members. Western blot analysis showed activation of ErbB2 and ErbB3, suggesting a possible mode of action of extracellular HRG in mammary gland carcinomas. We conclude that the extracellular and transmembrane domains of HRG are sufficient for the induction of tumorigenesis but that induction of apoptosis requires the cytoplasmic tail.

INTRODUCTION

The neuregulin family consists of over 15 splice isoforms arising from a single gene, *NRG1*. The protein products of these genes, including NDF[3], HRG, glial growth factors, and acetylcholine receptor inducing activity (1–4), and three additional genes [*NRG2*, *NRG3*, and *NRG4* (5–7)] interact with the EGFR members. Binding occurs primarily with ErbB3 or ErbB4 and leads to receptor homodimerization or heterodimerization with ErbB2 or EGFR.

EGFR amplification has been associated with breast, lung, stomach, and bladder cancer (8). Another member of the EGFR family, ErbB2/HER2, has also been implicated in a variety of human cancers. Amplification and overexpression of this gene is seen in approximately 30% of breast cancers (9) and is associated with higher chances of distant metastases and poor prognosis (10–12). Studies show that the newer members of this family, ErbB3 and ErbB4, may also be overexpressed in mammary tumors (13, 14).

The potential role of the various isoforms of neuregulin in regulation of normal and oncogenic signals through its receptor has been the subject of intense investigation (15). The effect of HRG on mammary gland cell lines can be either mitogenic or inhibitory and may be a function of the receptor types that are expressed on the surface of the cells (16, 17). Cell lines that have been shown to have high expression levels of ErbB2 show arrest and differentiation when treated with high concentrations of HRG but proliferate when treated with low levels of HRG (16). Mammary gland cell lines with low levels of ErbB2, however, appear to be growth-stimulated by treatment with HRG at high or low concentrations (18). Transgenic mice containing the HRG β2c isoform under transcriptional control of the MMTV promoter develop mammary gland tumors after 1 year of continuous mating and have extensive mammary gland hyperplasia (19).

The major motifs found in the various NRG1 isoforms include immunoglobulin-like *N*- and *O*-linked glycosylation regions and EGF-like transmembrane and cytoplasmic domains. The role that each domain plays in the mediation of signaling has not been fully elucidated. Recombinant EGF-like domain is sufficient to induce receptor activation (2), whereas other regions may function to regulate the multiple biological actions of HRG (20). HRG β2b was isolated in a screen for dominant factors involved in apoptosis. The cytoplasmic domain of this protein was found to be sufficient for induction of programmed cell death (21, 22). The cytoplasmic domain of HRG β2c was then also shown to mediate apoptosis in mammary gland epithelial cells (23). A new isoform, HRG γ, has been found that lacks transmembrane and cytoplasmic domains but still binds to ErbB3 and ErbB4, thus indicating that the cytoplasmic region is not necessary for the proliferative effects of the protein (24). This isoform has since been shown to be the product of a chromosomal translocation event fusing *DOC4*, a gene encoding for a secreted stress protein, to the *NRG1* gene (25).

Given the above-mentioned results, we sought to determine whether the extracellular and transmembrane regions of HRG would be sufficient to induce mammary gland carcinomas *in vivo*. We hypothesized that a transgenic mouse expressing the extracellular region of HRG transcribed from the MMTV promoter would develop mammary hyperplasia as well as mammary gland tumors. Furthermore, unlike tumors that arise in the MMTV/full-length HRG mouse, these tumors, and cell lines that are derived from them should not display high levels of apoptosis because the transgene lacks the cytoplasmic region.

MATERIALS AND METHODS

Mammary Gland Whole Mounts. Mammary glands were treated as described previously (19). Briefly, the inguinal mammary fat pads were excised and spread onto a glass slide. After overnight fixation in Tellyesniczky's fixative (70% ethanol:formaldehyde:glacial acetic acid at a 20:1:1 ratio) at room temperature, the samples were rehydrated and stained overnight in a carmine red solution (1 g of carmine red dye and 2.5 g of potassium alum in 500 ml of water). The tissues were then washed in water and slowly dehydrated in ethanol, followed by two overnight incubations in xylene.

Histological Evaluation of Tumors. Tumor samples were fixed in Optimal Fix (American Histology Reagent Company, Inc.). Five-μM sections were then cut, and the samples were stained with H&E by the Transgenic Core Pathology Laboratory at the University of California at Davis (Davis, CA).

Transgene Construction and Injection. An 831-bp fragment of the extracellular and transmembrane region of HRG was amplified by PCR from the

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[1] To whom requests for reprints should be addressed, at Department of Genetics, Harvard Medical School, Boston, MA 02115. Phone: (617) 432-7667; Fax: (617) 432-7944; E-mail: leder@rascal.med.harvard.edu.

[3] The abbreviations used are: NDF, neu differentiation factor; HRG, heregulin; MMTV, murine mammary tumor virus; TEB, terminal end bud; EGF, epidermal growth factor; EGFR, EGF receptor; poly(A), polyadenylic acid.

β2b isoform of a NDF expression construct described previously (21) using the primer pair of 5′-GGCAAGCTTGCCACCATGTCTGAGCGCAAAG-AAG-3′ and 5′-GCGAATTCGCCGGAGCCGATCATGAAGC-3′. The PCR product was digested with *Hin*dIII, ligated into a plasmid cassette containing the MMTV long terminal repeat and SV40 polyadenylation and splicing signals (26), and digested with *Bam*HI to check for insert orientation. The MMTV/HEX plasmid (MMTV/HEX) was then linearized with *Xho*I, run on a 1% agarose gel, and purified on a Qiagen column. Injection buffer (0.15 M KCl, 5 mM NaCl, and 10 mM PIPES) was subsequently used to dilute the DNA to a concentration of 1–10 ng/μl. The DNA solution was then injected into one of two pronuclei of a fertilized FVB/n egg and implanted into the oviduct of a pseudopregnant Swiss/Webster mouse.

Northern Blot Analysis. Poly(A) purified RNA was isolated from mammary gland or mammary tumor using RNA STAT-60 (Tel-Test, Inc.) followed by streptavidin magnetic particle separation (Boehringer Mannheim) and was size-fractionated on a 1.2% agarose gel with 1× 4-morpholinepropanesulfonic acid. The gel was transferred onto GeneScreen membrane (DuPont), and the filter was UV cross-linked and prehybridized in hybridization solution (50% formamide, 5× SSC, 5% dextran sulfate, 20 mM sodium phosphate, 1× Denhardt's reagent, 0.5% SDS, and 20 μg/ml sonicated herring sperm DNA) at 42°C for at least 1 h. An 800-bp *Pst*I fragment of SV40 poly(A) vector was labeled with [α-^{32}P]dCTP using Prime-It random priming (Stratagene) and hybridized to the membrane overnight at 42°C. Filters were washed at room temperature twice for 15 min in 2× SSC/0.1% SDS and once for 10 min in 0.1× SSC/0.1% SDS before exposing to film.

DNA Fragmentation Assay. Cells to be assayed were grown for 24 h in DMEM (Life Technologies, Inc.) with 10% fetal bovine serum (Sigma) and treated with 1 μM dexamethasone (Sigma) dissolved in 90% ethanol or with 90% ethanol alone. Cells were washed once with 1× PBS and detached from plates by scraping. Approximately 1×10^7 cells were lysed in cold lysis buffer [10 mM Tris-HCl (pH7.5), 10 mM EDTA, and 0.2% Triton X-100] and microfuged for 10 min at 4°C. The supernatant was phenol/chloroform extracted, and DNA was precipitated through the addition of 29 μl of 5 M NaCl and 980 μl of ethanol. The pellet was washed with 70% ethanol, resuspended in 15 μl of water, and treated for 30 min at 37°C with 2 μl of 10 mg/ml RNase A. The DNA was then electrophoresed through a 2% agarose gel in 1× TAE.

Immunoprecipitation and Western Blot Analysis. Cell lysates were produced by incubating cells for 10 min at 4°C in TNEN lysis buffer (27), and protein concentrations were determined with a Bio-Rad dye binding assay. Immunoprecipitations were performed using 1.0 mg of protein lysate with the indicated antibodies [Ab3 anti-neu monoclonal antibody (Oncogene Science), anti-ErbB3 and anti-ErbB4 rabbit polyclonal antibodies (Santa Cruz Biotechnology), and protein A/protein G chimera agarose (Pierce)]. Proteins were fractionated by running through 8% or 12% SDS-PAGE gels and transferred onto polyvinylidene difluoride membrane (Millipore). The filters were blocked for 1 h in 1× TBS with 0.1% Tween and 5% nonfat powdered milk and probed overnight in primary antibody in blocking buffer at 4°C. Incubation of the secondary antibody was performed at room temperature for 1 h. Signals were developed through use of the enhanced chemiluminescence method (Amersham).

***In Situ* Apoptosis Analysis.** Mouse tumors were dissected, fixed in Optimal Fix (American Histology Reagent), dehydrated, embedded in paraffin, and sectioned to 7 μm. Apoptosis was then detected using the ApoTag Peroxidase detection kit (Intergen) according to the instructions of the manufacturer and as described previously (28). Tissues were counterstained with 0.1% Fast Green (Fisher Scientific) in 70% ethanol.

RESULTS

Expression of the Transgenic Extracellular HRG Construct in the Mammary Gland and Mammary Gland Tumors. To direct expression of extracellular HRG to mammary epithelia, we created transgenic mice carrying a construct consisting of the MMTV long terminal repeat promoter joined to 831 bp of the HRG gene. This form of HRG contains the EGF-like, immunoglobulin-like, and transmembrane domains of HRG β2 isolated previously from 293 kidney epithelial cells (21) but lacks all but the first eight amino acids of the cytoplasmic region. SV40 splicing and polyadenylation signals make up the 3′ end of the construct. The construct was injected into the male pronucleus of a one-cell mouse embryo, and founders were identified by Southern blot analysis (data not shown). Seven founders were identified, and four lines established that passed the transgene in a Mendelian fashion.

Multiparous females from all four transgenic lines (called MMTV/HEX) develop mammary gland carcinomas at approximately 17 months of age (Fig. 1). These tumors, as well as adjacent mammary gland tissue, were checked for transgene expression by Northern blot analysis using a probe to the SV40 poly(A) region (Fig. 2*A*). The transgene from the MMTV/HEX mice is shown to be expressed in both the tumor and the nontransformed mammary tissue. RNA from a tumor of the MMTV/full-length HRG transgenic mouse is included as a positive control for expression.

Epithelial cell lines were established from both the MMTV/full-length HRG β2c isoform and MMTV/HEX mammary tumors. The cells were harvested, and lysates were run into a 12% SDS-PAGE gel. Probing the filter with a goat polyclonal antibody to HRG β (Santa Cruz Biotechnology) reveals an approximately M_r 21,000 band in the MMTV/HEX lane (Fig. 2*B*). The predicted size of the β2 isoform lacking a cytoplasmic domain is M_r 23,800. The full-length β2c isoform runs slightly higher than the predicted M_r 38,000, which can be accounted for by glycosylation. Protein lysates made directly from mammary gland tumors of MMTV/HEX mice reveal a M_r 21,000–23,000 band as well (data not shown).

Effect of the Extracellular HRG Transgene on the Virgin and Multiparous Murine Mammary Gland. The highest levels of transcription from the MMTV promoter are stimulated by pregnancy and lactation (29), although the transgene is expressed in the virgin mammary gland as well (19). We prepared mammary gland whole mounts from adult virgin females to determine whether there are any developmental abnormalities in the transgenic animals. In normal development, TEBs provide a source of ductal and myoepithelial cells, which allows for the elongation of secondary ducts as they move to the periphery of the fat pad (30). In the wild-type female mammary gland, TEBs undergo apoptosis and regression by 10 weeks of age (Ref. 31; Fig. 3*A*). Whereas the overall architecture of the transgenic animal is largely preserved, there are increased numbers of TEBs compared with wild-type littermates (Fig. 3*B*). In addition to the persistence of TEBs after the ducts have reached the margins of the fat pad, some

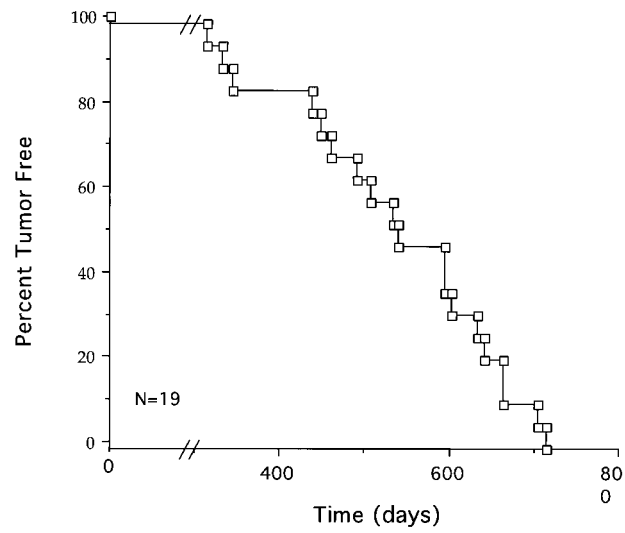

Fig. 1. Mammary gland tumor incidence in MMTV/HEX female mice. All mice 2 years of age and younger were evaluated for palpable mammary gland tumors. The number of mice represented is 19.

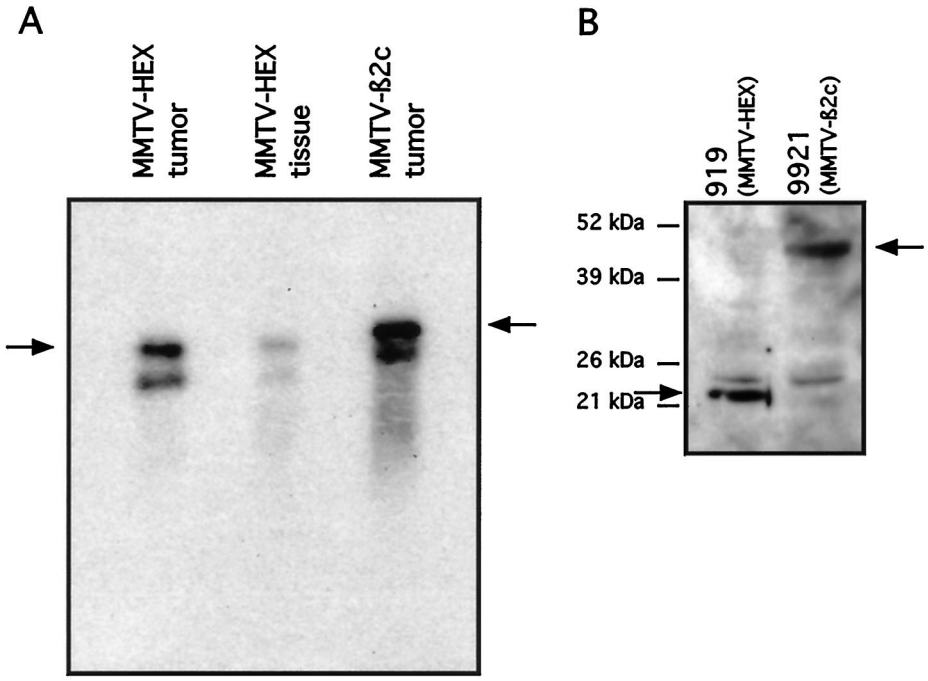

Fig. 2. Expression analysis of tissue, tumor, and cell line from a transgenic mouse carrying the MMTV/HEX transgene. *A*, total RNA was run on a 1.2% agarose gel and probed with a SV40 poly(A) fragment. Western blot analysis of expression of the extracellular transgene as compared with the full-length transgene in mammary tumor cell lines is shown. *B*, protein from whole cell lysate was run on a 12% SDS-PAGE. Primary antibody is against HRG β (C-18; Santa Cruz Biotechnology).

TEBs appear to have reversed direction of growth. Both of these phenotypes have been reported in MMTV/full-length β2c HRG transgenic female virgin mice (1).

The extracellular HRG transgene produces a mammary gland phenotype in multiparous females as well. The mammary gland of a wild-type female undergoes a short burst of secretory engorgement after weaning of a litter. This is followed by absorption of milk protein and collapse of alveoli (31). Whereas there may be some residual enlarged terminal ducts, the overall structure of the fully regressed FVB mammary gland closely resembles that of the adult virgin mammary gland by approximately 2 weeks after weaning (Fig. 3*C*). In contrast, the mammary gland from a multiparous MMTV/full-length HRG female shows considerable residual hyperplasia months after weaning (Fig. 3*D*). This phenotype is even more pronounced in the MMTV/HEX multiparous females (Fig. 3*E*). Even 6 months after weaning, these glands remain highly hyperplastic. At approximately 17 months of age, multiparous females develop mammary gland tumors, some of which appear histologically similar to tumors from the MMTV/neu mouse (Fig. 3*F*). They are intermediate cell nodular tumors with areas of extensive necrosis and regions that contain keratinizing epithelium filled with laminar keratin.

The HRG Extracellular Transgene Does Not Induce Apoptosis in Mammary Gland Cells. Having established that the extracellular region of HRG is sufficient to induce tumorigenesis in the murine mammary gland, we next investigated its ability to induce apoptosis. As noted previously, full-length HRG is capable, both *in vitro* and *in*

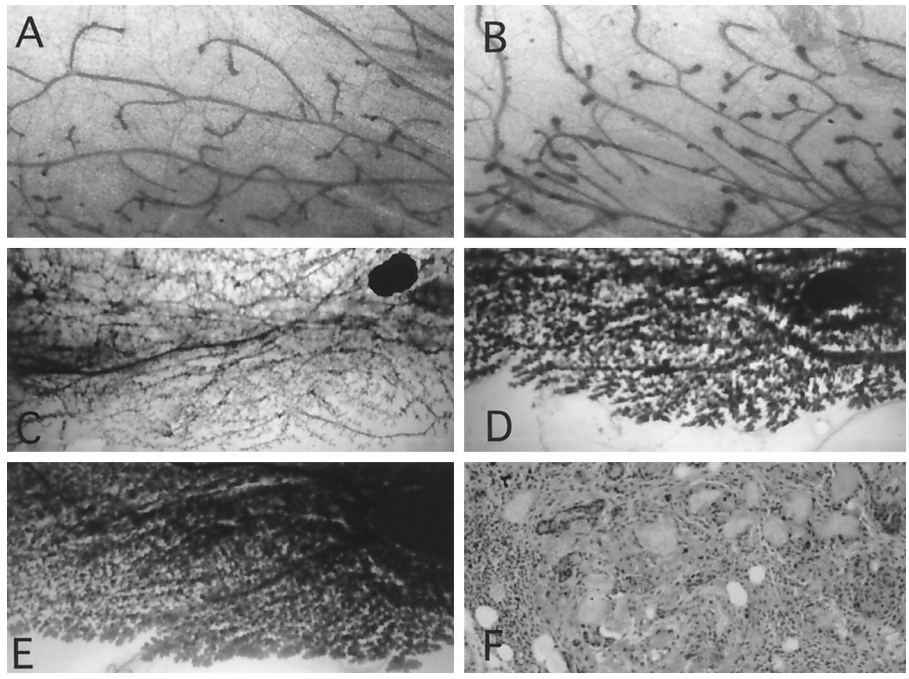

Fig. 3. Mammary gland whole mounts and histopathology. Persistence of TEBs in a 2-month-old nulliparous wild-type female (*A*) as compared with a transgenic littermate (*B*). Mammary gland of an adult multiparous wild-type female (*C*) compared with MMTV/HRG β2c transgenic adult multiparous mammary gland (*D*) and MMTV/HEX transgenic adult multiparous mammary gland (*E*). F, H&E-stained tumor section of a MMTV/HEX transgenic mammary gland.

vivo, of inducing both cell growth and programmed cell death in epithelial cells (22, 23). Because this data indicated the necessity of the cytoplasmic domain in driving apoptosis, it seemed doubtful that extensive cell death would occur in mammary cells overexpressing the extracellular HRG transgene. An epithelial cell line was established for a MMTV/HEX mammary tumor (MMTV/HEX 919) and analyzed for expression of the transgene (Fig. 4*A*, *Lane 1*). Because the expression level for this transgene was low, dexamethasone was used at various concentrations to induce the MMTV promoter (32). At a final concentration of 1 μM, dexamethasone induced transgene expression to a level roughly comparable with that seen in the MMTV/HRG β2c epithelial line 9921 (Fig. 4*A*, *Lanes 2-4*). A DNA laddering assay was then performed on these cells to determine whether apoptosis was occurring (Fig. 4*B*). When the DNA is run on a 2% agarose gel, it can be seen that the MMTV/HRG β2c cell line is undergoing programmed cell death as expected, whereas the cell lines overexpressing the MMTV/HEX transgene are not undergoing programmed cell death.

Because cell lines can undergo and accumulate mutations, we sought to directly analyze apoptosis in sections of the mammary gland tumors. The terminal deoxynucleotidyl transferase-mediated nick end labeling assay was used, in which double-stranded DNA breaks indicative of apoptosis are identified by the enzyme terminal deoxynucleotidyl transferase. Multiple tumors isolated from the mammary glands of MMTV/HEX transgenic animals fail to show significant levels of apoptosis, whereas those from the MMTV/HRG β2c mice are clearly spotted with cells undergoing programmed death (Fig. 4*C*). MMTV/Ras tumors were used as a negative control for nonspecific staining.

EGFR Family Expression in Mammary Tumors Induced by the MMTV/HRG Extracellular Domain Transgene. Tumors induced by the β2c isoform of HRG have already been shown to express ErbB3 and ErbB4, whereas only ErbB3 was phosphorylated (19). Antibodies against ErbB2, ErbB3, and ErbB4 were used to immunoprecipitate protein from lysates of the MMTV/HEX and MMTV/HRG β2c cell lines. The immunoprecipitated proteins were then subjected to analysis by Western blot with an antiphosphotyrosine antibody (4G10; Upstate Biotechnology). Consistent with previous results, only ErbB3 was phosphorylated in the cell line established from the full-length HRG transgenic tumor (Fig. 5*A*). There is, however, a high

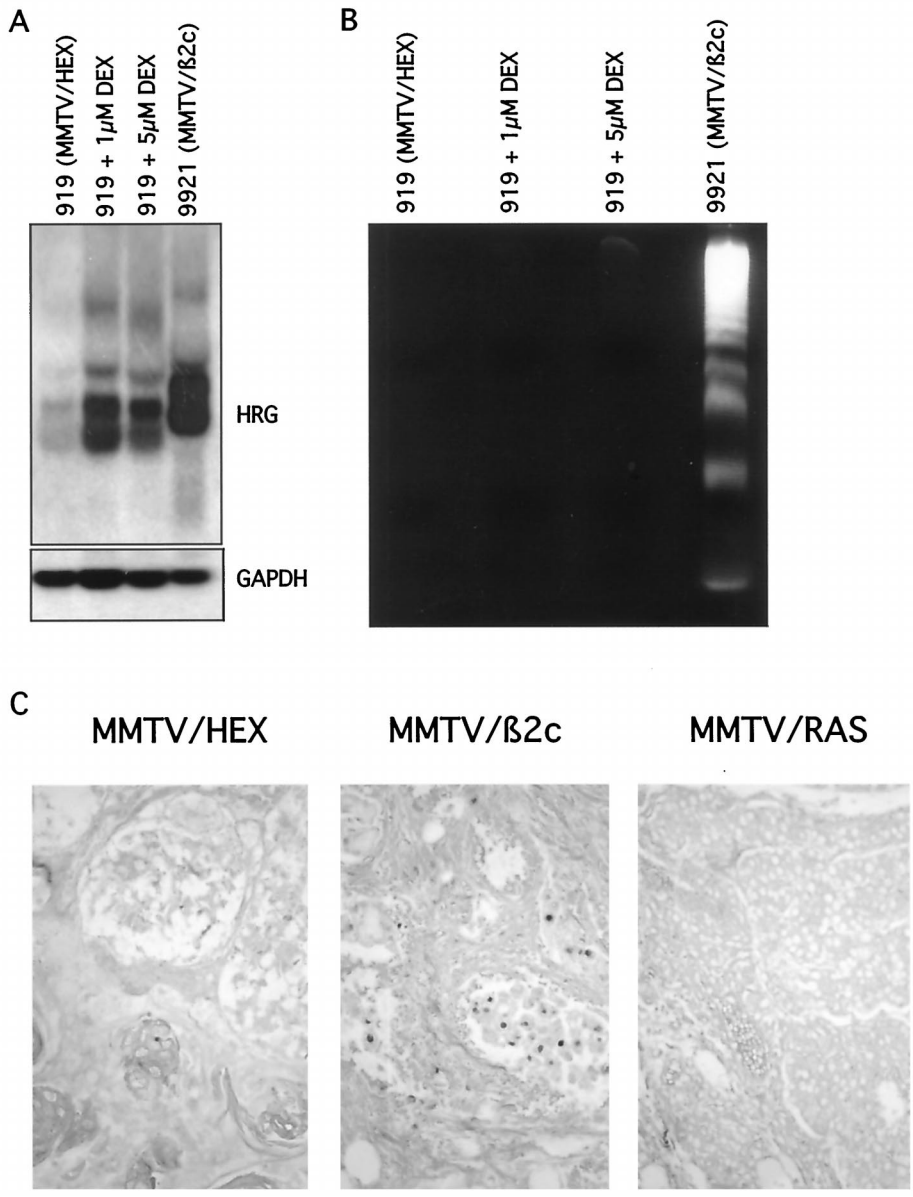

Fig. 4. Absence of apoptosis in mammary gland tumors of MMTV/HEX domain mouse. *A*, expression of the transgene in cell line with or without stimulation by dexamethasone, as determined by probing a Northern blot with a probe for the SV40 poly(A) tail. *B*, DNA fragmentation analysis of cell lines harvested and lysed in hypotonic buffer and analyzed on a 2% agarose gel in 1× TAE. *C*, *in situ* apoptosis detection in mammary gland tumors from MMTV/HEX domain, MMTV/HRG β2c, and MMTV/RAS transgenic mice.

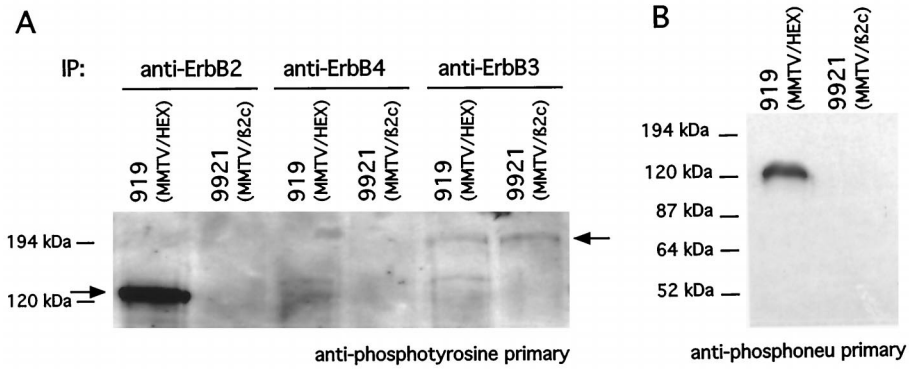

Fig. 5. Immunoblot analysis of mammary gland tumor cell lines. *A*, MMTV/HRG β2c and MMTV/HEX domain tumor cell lysates were prepared, and immunoprecipitation was performed with EGFR family antibodies. The filter was probed with an anti-phosphotyrosine antibody (Upstate Biotechnology). *B*, whole cell lysates of both cell lines are run on an 8% gel and probed with anti-activated neu (Upstate Biotechnology).

level of phosphorylated ErbB2 in the cell line from the MMTV/HEX mammary tumor, in addition to the presence of phosphorylated ErbB3. This ErbB2 band runs at about M_r 120,000, which is significantly smaller than the expected M_r 185,000. To show that this result is not an artifact associated with this antibody, whole cell lysate was run on an 8% acrylamide gel, and a different primary antibody was applied (anti-phosphorylated neu; Upstate Biotechnology). Again, the band is seen in the MMTV/HEX lane but not in the MMTV/HRG β2c lysate (Fig. 5*B*). Various antibodies to ErbB2 showed the M_r 120,000 band to be the only form of ErbB2 present (data not shown).

DISCUSSION

A wide range of transgenic mice have been created to demonstrate that activation of ErbB2 leads to mammary gland carcinogenesis. Mice that overexpress neu with an activating point mutation (26) as well as mice that express the cellular form of neu (33) both develop tumors. Overexpression of the β2c isoform of HRG, one ligand of the EGFR family of transmembrane tyrosine kinases, has also proven potent in the induction of mammary carcinomas (19). The cytoplasmic domain of this isoform, however, was shown to be involved in programmed cell death rather than cellular proliferation (22, 23). We hypothesized that overexpression of the extracellular and transmembrane domain of HRG should be sufficient for tumorigenesis. The results of our experiments support this notion in that all four transgenic lines carrying the MMTV/HEX construct did develop mammary gland carcinomas at approximately 17 months of age.

By examination of premalignant mammary glands, we could see the persistence of TEBs in postpubescent virgin female mice. Under normal conditions of development, TEBs provide the source of myoepithelial cells for the elongating mammary ducts. The myoepithelial cells receive signals from the mesenchyme when the outer limits of the fat pad have been reached, which leads to cessation of growth and elongation of the ductal structures. The TEBs would normally undergo apoptosis at this time. However, the mammary glands in our transgenic mice carrying the MMTV/HEX construct show disorganization and persistence in the TEB structure, indicating that signaling between stromal and epithelial cells is not progressing as usual. This phenotype is seen in transgenic mice bearing the full-length MMTV/HRG β2c construct (19) as well. Whatever signaling HRG is mediating in this capacity is overcome by pregnancy because TEB persistence is not seen in the fully regressed mammary gland. Therefore, persistence of proliferating cells within the TEBs may offer an additional target for malignant transformation that is eliminated when pregnancy occurs. In the rat mammary gland, for example, sensitivity to applied carcinogens is at its maximum level during the TEB growth phase (34).

Both MMTV/HEX and MMTV/HRG β2c transgenic mice show hyperplasia of the mammary gland after multiple pregnancies. The mammary gland of the truncated HRG transgenic mouse shows a profound increase in hyperplasia when compared with that of the wild-type littermate and the MMTV/HRG β2c animal. It is possible that the mammary gland of the MMTV/HRG β2c transgenic mouse is experiencing increased signals for cell proliferation due to the activity of the extracellular domain of HRG, but that growth is tempered somewhat by the apoptosis-inducing cytoplasmic region of the protein. However, the protein produced by the MMTV/HEX mouse lacks the cytoplasmic domain and therefore lacks the ability to simultaneously induce apoptosis and growth. From these results, it would be reasonable to hypothesize that a transgenic mouse expressing only the cytoplasmic tail of HRG in the mammary gland could be mated to other oncogene-expressing mice, resulting in a longer latency of tumorigenesis.

We further confirmed that the extracellular region of HRG was sufficient to induce tumors but not apoptosis through examination of mammary gland tumors and cell lines. Whereas MMTV/HEX animals do develop mammary gland carcinomas, neither these tumors nor the cell lines derived from them show high levels of apoptosis. This is in contrast to tumors and cell lines from the MMTV/HRG β2c mice. This direct comparison demonstrates not only that the extracellular plus transmembrane domains of HRG are sufficient to bring about mammary gland tumors but also that the cytoplasmic region is necessary for HRG-mediated programmed cell death *in vivo*.

The receptors for HRG, ErbB2, ErbB3, and ErbB4, have all been shown to be endocytosis impaired (35). When overexpressing their ligand by a strong promoter such as MMTV, one would expect that some combination of the ErbB receptors would show activation in the form of phosphorylation. Previous work with mammary gland tumors derived from the MMTV/full-length HRG β2c transgenic line showed tyrosine phosphorylation of ErbB3 but not of ErbB2 or ErbB4 (19). It is unlikely that signaling occurs through ErbB3 homodimers because they are believed to be kinase inactive (36, 37), although there is the possibility that ErbB3 is dimerizing with an as yet undescribed ErbB receptor. In the MMTV/HEX mice, however, both ErbB2 and ErbB3 are shown to be phosphorylated in the mammary gland tumor. These data are consistent with previous tissue culture experiments showing mediation of the effects of HRG through ErbB2/ErbB3 dimerization (15, 38) and suggest a possible mechanism by which the mammary gland is transformed in this transgenic line.

Whereas overexpression of the extracellular HRG did lead to phosphorylation of ErbB2, the protein is clearly truncated. Multiple Western blots with various antibodies confirmed this result (data not shown). The antibodies that hybridized to the approximately M_r 120,000 protein band were all directed to the COOH terminus of ErbB2. NH_2-terminally truncated forms of ErbB2 have been identified

previously (39), and expression of the truncated protein has been correlated with node-positive breast cancer (40). Shedding of the extracellular domains of ErbB2 has also proven to interact with anti-ErbB2 antibodies and to have an inhibitory effect in a therapeutic setting (41). It would therefore be worthwhile to assess HEX domain-induced mammary tumor cell lines for the soluble form of the receptor.

A recent report has shown that the isoform of NDF known as HRG γ is actually the product of a chromosomal translocation fusing the secreted DOC4 protein to the extracellular region of HRG (25). Whereas this chimeric protein is seen in MDA-MB-175 breast cancer cells, reverse transcription-PCR analysis showed it to be absent from a large number of breast carcinoma samples and cell lines, indicating that this particular translocation is not a common event in breast cancer. However, our results show that the extracellular/transmembrane region of HRG is sufficient to induce breast carcinomas and justifies examination of ErbB2-positive tumors for the presence of HEX domain overexpression.

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