The Matrix Metalloproteinase Matrilysin Influences Early-Stage Mammary Tumorigenesis

Laura A. Rudolph-Owen, Richard Chan, William J. Muller, and Lynn M. Matrisian

ABSTRACT

Overexpression of the epithelial specific matrix metalloproteinase matrilysin (MAT) has been correlated with enhanced tumorigenicity and tumor cell invasion using in vitro model systems. We have determined the effects of MAT expression on the development of mammary tumorigenesis using transgenic mice that express human MAT under the control of the mouse mammary tumor virus (MMTV)-long terminal repeat promoter/enhancer. Examination of mammary glands from multiparous MMTV-MAT animals revealed the development of premalignant hyperplastic alveolar nodules in 50% of aged females. MMTV-MAT mice were mated with MMTV-neu transgenic mice to determine the effect of MAT on neu-induced mammary tumorigenesis. Bigenic MMTV-MAT/neu female offspring developed primary mammary tumors ~13 weeks earlier than did MMTV-neu controls. The mechanism of enhanced neu-induced tumor formation was explored. No discernible difference in Neu receptor dimerization or activation was detected in MMTV-MAT/neu tumors or mammary glands compared to MMTV-neu controls. A similar percentage of MMTV-MAT/neu and MMTV-neu tumors acquired deletions in the neu transgene, which have previously been shown to result in constitutive receptor activation. The presence of premalignant nodules and the accelerated development of oncogene-induced mammary tumors suggest that expression of MAT in the mammary epithelium contributes to early-stage mammary tumorigenesis.

INTRODUCTION

Breast cancer is the leading cause of mortality due to cancer among nonsmoking women in the United States (1). Lethality is usually the result of local invasion and metastasis of neoplastic cells from the primary tumor into the underlying stroma, entry into the circulation, and growth of the cancer cells at distant sites in the body (2). Because of their ability to degrade extracellular matrix components, the MMPs have been implicated in the breakdown of basement membrane and underlying stroma, thereby facilitating tumor growth, invasion, and metastasis. A causal role for MMPs in these processes has been established by studies using natural and synthetic inhibitors of MMPs (Ref. 3 and references therein).

Increased expression of MMPs has been detected in various forms of mammary disease, correlating MMP expression with advancement of tumor stage (reviewed in Ref. 3). Of the MMP family members examined, MAT (MMP-7, pump-1, and uterine metalloproteinase; EC 3.4.24.23) is distinctive in that it is the only MMP that is expressed almost exclusively in the epithelial component of the tumor, compared to the predominantly stromal expression of other MMP family members. MAT mRNA was detected in the neoplastic epithelial tumor cells of 70–91% of breast adenocarcinomas (4–6). The expression of MAT in the malignant epithelium of the colon, prostate, stomach, and lung (7) makes it an ideal candidate to contribute to the invasive and metastatic phenotype of these tumors. Indeed, the overexpression of MAT in prostate tumor-derived cell lines enhances the ability of these cells to invade the diaphragm of immunodeficient mice (8). However, MAT mRNA has been observed in benign breast fibroadenomas (9) and in a high percentage of ductal carcinoma in situ specimens (6), neither of which demonstrate invasive properties. MAT mRNA and MAT protein have also been detected in nonmalignant breast epithelium (6, 9) and at low levels in the mammary glands of adult cycling female mice (10). Because MAT is expressed in normal, benign, and malignant mammary tissues, the presence of the MAT protein is apparently not sufficient for tumor cell invasion. In the colon, MAT is expressed in a high percentage of adenomas (11), and the overexpression of MAT in colon-derived cell lines enhances tumorigenicity but not necessarily metastasis, following orthotopic injection into nude mice (12). In addition, we have recently demonstrated that the lack of MAT in a murine model of familial adenomatous polyposis reduces the incidence of benign lesions (13). Thus, MAT in particular may play a role in early stages of tumor progression, in addition to contributing to late-stage tumor invasion and metastasis.

neu/ErbB-2 amplification has been observed to be amplified and overexpressed in a significant number of human breast cancers (14). Neu signaling is dependent on heterodimerization with other ligand-binding ErbB receptor family members because Neu does not directly bind ligand (reviewed in Ref. 15). Members of the EGF family of ligands, including EGF, TGF-α, HB-EGF, amphiregulin, and betacellulin, can transmit this signal through their association with the EGFR. Similarly, binding of the heregulin family of ligands to ErbB-3 or ErbB-4 can also transmit a mitogenic signal. Several studies have shown that a high degree of neu/ErbB-2 amplification is correlated with a poor clinical outcome (14, 16). Because of this close correlation between neu overexpression and mammary carcinogenesis, transgenic mice that carry the native Neu protein under the control of the MMTV-long terminal repeat promoter were generated to test the oncogenic potential of neu in mammary epithelium directly. Overexpression of the neu product in the mammary epithelium resulted in the appearance of focal mammary adenocarcinomas in ~70% of multiparous females by an average of 205 days that metastasized to the lungs in 72% of tumor-bearing animals (17). Although the enhancing effects of pregnancy do not accurately recapitulate the human disease, the pathological features and metastatic potential of these tumors appropriately mimic the majority of human breast cancers (18). The development of mammary tumors in the MMTV-neu transgenic animals was found to be caused by various deletions within the neu transgene, which, although these same deletions have not yet been described in human tumors, result in the same end point of constitutive activation of the Neu signal transduction pathway (19). Thus, the MMTV-neu transgenic mice represent a reasonable model of human breast cancer,
based on the causative agent, the pathology, and the progression of the disease (18).

Here, we attempted to recapitulate the expression of MAT in human breast in a mouse model system to determine the contribution of this MMP to various stages of mammary tumor progression. MAT was targeted to normal mammary epithelium under the control of the MMTV-long terminal repeat. The expression of MAT in the mammary gland had no effect on ductal branching, although production of the milk protein β-casein was observed in virgin mice (20). The MMTV-MAT mice show no evidence of developing palpable breast nodules in virgin or multiparous aged females. Therefore, to place MAT in the context of malignant breast epithelium, MMTV-MAT transgenic mice were mated with MMTV-neu animals and bigenic female offspring analyzed for mammary tumor development. Interestingly, MAT greatly enhanced the onset of primary mammary tumors, suggesting that MAT has a more extensive role in early tumor development than previously assumed. In addition, we explore the possibility that the tumor-enhancing effect of MAT is mediated through a Neu-related signaling pathway.

**MATERIALS AND METHODS**

**Animal Models and Tumor Assessment.** Transgenic animals expressing wild-type human MAT (line 3; Ref. 20) were mated to transgenic animals expressing wild-type rat c-neu (line 22; Ref. 17), both under the control of the MMTV promoter/enhancer. The MAT transgene mRNA levels in the murine mammary gland were detectable by Northern blot analysis and were therefore roughly in the same range as that observed in fibroadenomas analyzed by the same technique (5). In addition, MAT protein was detectable by immunohistochemistry in both the transgenic mice and in human breast reduction samples (9, 20), although this technique is not sufficiently quantitative to comment on the relative levels of MAT expression. Both transgenic lines were originally generated in the FVB strain of mice so that the genetic background of the parental and the double transgenic animals were identical. The resulting offspring were assayed for the inheritance of both transgenes by Southern blot analysis of genomic tail DNA using a random-primed (DNA Labeling Kit; Boehringer Mannheim, Indianapolis, IN) 1.1-kb Xbal/EcoRI fragment of human MAT (pG7pumpEX; Ref. 21) and a 795-bp BamHI fragment of rat c-neu (pMMTV-neu; Ref. 17).

Female transgenic animals were tested for the presence of mammary tumors by weekly palpitation, beginning at ~18–20 weeks of age. Thereafter, tumors were measured with a caliper in two dimensions (length and width) on a weekly basis to monitor tumor growth rate. The formula length/2 X (width)2 was used to determine the approximate volume of an elliptical tumor. The total volume of each mammary tumor was plotted against days of tumor growth, and the approximate doubling time of each tumor extrapolated from the graph. Mice were sacrificed before tumor size reached 20 mm in diameter. Whole mount analysis for the detection of HANs was performed on the thoracic (second and third) and inguinal (fourth) mammary glands on the left side of each animal, as described previously (22).

**Antibodies.** Affinity purified antihuman MAT polyclonal antibody was a kind gift from Dr. William Parks (Washington University School of Medicine, St. Louis, MO; Ref. 9). Rabbit polyclonal antibody to mouse casin was kindly provided by Dr. Charles Daniel (University of California at Santa Cruz, Santa Cruz, CA; Ref. 23). Polyclonal antibodies against EGF-R (1005), Neu (C-18), ErbB-3 (C-17), and ErbB-4 (C-18) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibodies to phosphotyrosine were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Immunohistochemistry.** Paraformaldehyde-fixed, paraffin-embedded sections were analyzed as described previously (20). Sections were incubated overnight at 4°C in blocking solution with antihuman MAT primary antibody (1:1000 dilution) or control rabbit IgG (Sigma Immunochemicals, St. Louis, MO) and then incubated with biotinylated goat IgG (1:5000; Vector Laboratories, Burlingame, CA) for at least 1 h at room temperature. Labeled cells were visualized using an avidin-biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories) and TrueBlue peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Sections were then counterstained with Contrast Red.

**Immunoprecipitation and Western Blot.** For analysis of total cell lysates, frozen tissue was pulverized with a mortar and pestle, homogenized in ice-cold protein lysis buffer [50 mM Tris (pH 8.0), 100 mM NaCl, 20 mM HEPES, 1% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride], and incubated on ice for 20 min with occasional vortexing. Insoluble material was removed by centrifugation at 4°C, and the supernatant was collected. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA). To precipitate specific ErbB receptors in isolation, 200–500 μg of cellular protein were boiled for 5 min in boiling buffer [0.5% SDS, 1 mM DTT, and 50 mM Tris (pH 7.4)] to dissociate preformed receptor complexes and then placed on ice. Alternatively, protein lysates are directly immunoprecipitated without boiling for detection of ErbB receptor complexes. Approximately 0.5 μg of ErbB-specific polyclonal antibody was then added and incubated overnight at 4°C, followed by a 1-h incubation at 4°C with protein A-Sepharose CL-4B (Pharmacia Biotech, Piscataway, NJ). Immune complexes were washed five times with protein lysis buffer, resuspended in 2X sample buffer [4% SDS, 200 mM DTT, 120 mM Tris (pH 6.8), 10% glycerol, and 0.02% bromphenol blue], and boiled for 10 min. Samples were electrophoresed through a SDS-8.0% polyacrylamide gel and transferred to nitrocellulose membranes (MSI, Westboro, MA) for Western blotting. Membranes were blocked for at least 1 h at room temperature with TBST buffer containing 2.5% nonfat dried milk. Phosphotyrosine (1 μg/ml) or ErbB (1:10,000 dilution) antibody was added for 2 h at room temperature with rotation. Membranes were washed with TBST buffer and incubated with an antirabbit, horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI; 1:40,000 dilution) for 1 h at room temperature with rotation. The membranes were then washed in TBST and visualized by chemiluminescence. If necessary, blots were stripped [62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50°C] and reprobed with another primary antibody.

**RNAse Protection Assay.** Mammary glands and mammary gland tumors were homogenized in a guanidium/acid phenol solution, and total RNA was extracted as described by Chomczynski and Sacchi (24). Antisense riboprobes and phosphoglycerate kinase internal control probe was generated as described previously (19). RNAse protection assays were performed by hybridizing the above probes to 20 μg of total RNA (25). The protected fragments were separated on a 6% denaturing gel and subjected to autoradiography. The DNA markers correspond to Haell digest of X174 molecular weight standards (Life Technologies, Inc.), which were end labeled with γ-32PdATP.

**RESULTS**

Alterations in Multiparous MMTV-MAT Mammary Glands. Overexpression of the MAT transgene in MMTV-MAT transgenic female mice does not produce any observable morphological changes during mammary gland development (20). However, careful examination of whole mounts of representative mammary glands showed that four of eight (50%) aged, multiparous MMTV-MAT wild-type transgenic females contained abnormal structures in the mammary glands (Fig. 1A), whereas age-matched and pregnancy-matched nontransgenic mammary glands were devoid of such structures (zero of eight mice; P = 0.04 by Fisher’s exact test; Fig. 1B). These distinctive focal areas of epithelial hyperplasia strongly resemble structures previously termed HANs, which are considered to be premalignant precursors that are prone to develop into mammary carcinomas (26, 27). We have not observed the appearance of palpable mammary tumors in any MMTV-MAT transgenic animal after 3 years of observation. The appearance of HANs in the MMTV-MAT mammary glands suggests that overexpression of MAT predisposes the mammary gland to the formation of preneoplastic lesions but is not sufficient for the development of advanced mammary disease.

**Induction of Mammary Tumors in the MMTV-MAT Transgenics.** To investigate a potential role for MAT in mammary tumorigenesis, we induced mammary tumors in MMTV-MAT transgenic mice by mating them with MMTV-neu animals. The mammary glands of

5501
MMTV-MAT/neu and MMTV-neu transgenic offspring were palpated weekly to determine the onset of mammary tumors. Bisgenic MMTV-MAT/neu animals developed mammary tumors with a morphological and histological appearance similar to those previously reported in MMTV-neu single transgenic animals and typical of human breast adenocarcinomas (Ref. 17; Fig. 2, A and B). Histological examination of lung tissue from affected animals frequently revealed the presence of multiple nodular lesions lodged in pulmonary vessels (Fig. 2C). These lesions were verified as metastases originated from mammary tumors by the presence of β-casein immunoreactivity.4

MMTV-MAT/neu female transgenic mice (line 3) express detectable human MAT protein throughout the epithelium of developing (weeks 6–14) and adult mammary glands (20). The presence of the protein produced from the MAT transgene in the MMTV-MAT/neu mammary tumors was confirmed using an anti-MAT antibody that reacts with human but not mouse MAT (20). The MAT protein product was detected in isolated groups of cells lying at the periphery of MMTV-MAT/neu mammary tumors and not in tumors derived from MMTV-neu only transgenic mice (Fig. 2D). No endogenous murine MAT mRNA was detected in approximately six samples of MMTV-neu mammary tumors analyzed by Northern blot.4

The total number of mammary tumors per animal was similar in MMTV-MAT/neu compared to MMTV-neu animals (Table 1). However, we observed a dramatic acceleration in tumor onset in MMTV-MAT/neu mice compared to the MMTV-neu control animals (Fig. 3; P < 0.00001 by log rank test). Fifty % of female bisgenic animals developed mammary tumors by ~27 weeks, whereas 50% of single transgenic animals developed mammary tumors by ~40 weeks. In addition, 100% of the MMTV-MAT/neu double transgenic females formed mammary tumors by 40 weeks of age, whereas 20% of the neu females were still tumor free by 60 weeks of age (P = 0.05 by Fisher’s exact test; Fig. 3). Thus, the overexpression of MAT in neu-expressing mammary glands enhanced tumorigenesis by increasing the proportion of animals with tumors and shortening the time of tumor onset by an average of 13 weeks.

Mammary tumor growth was also monitored weekly by measuring the tumors with a caliper in two dimensions. The average doubling time of the MMTV-neu tumors was not significantly different from that of the MMTV-MAT/neu tumors (Table 1). These data indicate that, although the double transgenic mice developed mammary tumors earlier, the rate of tumor growth once established was similar between the two groups of animals.

To test the possibility that overexpression of MAT increases the metastatic ability of the MMTV-neu mammary tumor cells, we determined the percentage of double transgenic and single transgenic animals with secondary lung metastases. Eighty % of the MMTV-neu animals developed lung metastases, whereas 91% of the MMTV-MAT/neu double transgenic animals developed lung metastases (Table 1). Thus, the overexpression of MAT in the MMTV-neu animals resulted in no statistically significant increase in the metastatic ability of the mammary tumor cells.

**Function of Growth Factor Receptors in the Induction of Mammary Tumors.** The dramatic effect of MAT on mammary tumorigenesis raised the question of the mechanism by which MMP activity accelerates neu-induced mammary tumor formation. Similarly to our studies, the MMTV-TGF-α/neu mice (28) also developed mammary tumors earlier than the MMTV-neu transgenics. However, unlike the MMTV-neu (19) mammary tumors, the MMTV-TGF-α/neu (28) tumors do not contain deletions within the neu transgene, presumably due to the excess ligand available to constitutively activate the Neu signal transduction pathway. Because of the similar accelerated mammary tumor growth patterns of the MMTV-TGF-α/neu and the MMTV-MAT/neu animals, we proposed that a mechanism similar to that observed in the MMTV-TGF-α/neu animals may be operating in our system. Therefore, we hypothesized that MAT activity could result in an increase in the availability of soluble ErbB receptor ligands, through either cleavage of membrane precursors or release of ligand from matrix components, thereby constitutively activating the Neu signal transduction pathway. The processing of TGF-α (29) and HB-EGF (Ref. 30 and references therein) to their soluble forms is mediated by MMPs, providing support for this hypothesis. In addition, several EGFR ligands, including HB-EGF and amphiregulin, have high affinity for proteoglycans (31, 32), and MAT is a potent proteoglycanase (33). To test this hypothesis, we first analyzed the ability of Neu to heterodimerize and activate other ErbB receptor family members in MMTV-MAT/neu and MMTV-neu mammary tumors.

Antibodies specific to the EGFR, ErbB-3, and ErbB-4 were used to immunoprecipitate these receptors from mammary tumor protein lysates, either as complexes or isolated molecules. The presence of Neu within pre-existing cellular complexes was then analyzed by Western blotting. Immunoprecipitation with anti-EGFR and subsequent blotting for Neu revealed that Neu protein coimmunoprecipitated with the EGFR (Fig. 4A for representative samples). In addition, Neu was shown to coimmunoprecipitate with ErbB-3 and ErbB-4 in the same mammary tumor protein lysates.4 However, there was no discernible difference in the association of Neu with other family members receptors in the MMTV-MAT/neu mammary tumors compared to the MMTV-neu tumors.

To determine whether signaling through ErbB receptors occurred, phosphotyrosine (p-Tyr) levels of these proteins was analyzed by immunoprecipitation with antireceptor antibody and Western blotting.
Fig. 2. Histological appearance and MAT expression in MMTV-MAT/neu mammary tumors: a typical H&E-stained section of a mammary tumor from a MMTV-MAT/neu animal, viewed with a ×16 (A) and a ×50 (B) objective. C, a lung metastasis from a MMTV-MAT/neu animal with mammary tumors (×11 objective). Several metastases were usually noted in each lung. D, immunolocalization of human MAT in the MMTV-MAT/neu mammary tumors to the periphery and border of the tumor (arrows). T, primary mammary tumor (×50 objective).

Table 1 MMTV-MAT/neu versus MMTV-neu mammary tumor characteristics

<table>
<thead>
<tr>
<th>Transgenic animal</th>
<th>Tumor onseta (week)</th>
<th>No. of tumors per animalb</th>
<th>Doubling of tumors (days)</th>
<th>% lung metastasisb</th>
</tr>
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<tr>
<td>MMTV-MAT/neu</td>
<td>27</td>
<td>2 ± 1</td>
<td>15.5 ± 6.7</td>
<td>91 (10/11)</td>
</tr>
<tr>
<td>MMTV-neu</td>
<td>40</td>
<td>2 ± 1</td>
<td>14.0 ± 6.5</td>
<td>80 (12/15)</td>
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a Median values representing curves that are significantly different as determined by the log rank test; P < 0.00001.

b Not statistically different, as evaluated by the Student's t test.

with anti-p-Tyr (see Fig. 4, B and C, for representative samples). The EGFR was present and phosphorylated at moderate levels in both the MMTV-MAT/neu and MMTV-neu mammary tumor extracts (Fig. 4B). High levels of Neu were also found in mammary tumors and were associated with high levels of p-Tyr in both sets of tumors (Fig. 4C). ErbB-3 and ErbB-4 were also detected within the mammary tumor extracts but were associated with very low or undetectable levels of p-Tyr. These data illustrate that the EGFR and Neu are the only ErbB receptor family members that were activated at relatively consistent levels within the mammary tumor extracts. Importantly, the level of activation of the ErbB receptors was similar between the MMTV-MAT/neu tumors and the MMTV-neu tumors, suggesting that MAT has no effect on the levels of ErbB receptor signaling in fully developed mammary tumors.

Phosphorylation and activation of the ErbB receptors could theoretically also occur before the development of the mammary tumors. Mammary glands from MMTV-MAT/neu and MMTV-neu virgin animals at 25–30 weeks of age that were free of mammary tumors were processed, and protein extracts were examined for the presence of ErbB receptors and levels of p-Tyr. These data revealed little difference between the expression levels of the ErbB receptors, or their levels of p-Tyr between the MMTV-MAT/neu and the MMTV-neu virgin, tumor-free mammary glands.

Constitutive activation of neu by small deletions in the juxtamembrane domain has been demonstrated to contribute to the development of spontaneous mammary gland tumors observed in the MMTV-neu animals (19). Examination of the neu transgene by RNA protection revealed deletions in four of seven (57%) of our MMTV-MAT/neu mammary tumor samples, whereas uninvolved mammary glands lacked any neu alterations (Fig. 5). Similarly, MMTV-neu single transgenic mice develop altered neu message in 65% of the mammary tumors (18).

DISCUSSION

The experiments presented herein were designed to determine whether MAT expression can contribute to mammary tumorigenesis. Human MAT protein was targeted to normal mammary epithelium, mimicking the expression of MAT in normal-appearing breast epithelium obtained from breast reductions or surrounding malignant breast lesions (6, 9). Long-term exposure of transgenic mammary epithelium
MATRILYSIN ACCELERATES MAMMARY TUMORIGENESIS

A.

IP: EGFR

<table>
<thead>
<tr>
<th>Blot</th>
<th>Neu</th>
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<tr>
<td>MMTV-MAT/neu</td>
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N.S.

B.

IP: EGFR

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<th>Blot</th>
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<td>MMTV-MAT/neu</td>
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N.S.

C.

IP: Neu

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<td>MMTV-MAT/neu</td>
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p-Tyr

Fig. 4. ErbB receptor expression in the MMTV-MAT/neu and MMTV-MAT mammary tumors. A. coimmunoprecipitation of the ErbB family of receptors in MMTV-MAT/neu and MMTV-neu mammary tumor extracts. Immunoprecipitation of the EGFR, followed by Western blot for the Neu protein. B and C, immunoprecipitation and Western blot analysis of ErbB receptors in mammary tumor extracts from MMTV-MAT/neu and MMTV-neu animals. Each ErbB receptor [EGFR (B) and Neu (C)] was specifically immunoprecipitated from 300 μg of mammary tumor extracts and then Western blotted and analyzed for the levels of phosphorylation (p-Tyr), or for the presence of the immunoprecipitated receptor to control for the loading of protein. Five to eight mammary tumor samples were assayed, and four representative samples are shown for each group. N.S., nonspecific band.

To MAT expression resulted in lesions with a strong morphological resemblance to preneoplastic HANs in aged, multiparous females, although MMTV-MAT tissue has not been serially transplanted to determine the potential for neoplastic conversion. Previous studies suggest that the HAN is probably derived from a single cell but that an individual HAN population can undergo genetic changes that result in a biologically heterogeneous population of hyperplastic cells (reviewed in Ref. 27). HANs are susceptible to carcinogens, and exposure to chemical carcinogens, viruses, radiation, or exogenous hormones increases the tumor incidence of the hyperplastic cells and usually decreases the tumor latency period (27). Similarly, mating the MMTV-MAT animals to the MMTV-neu transgenics revealed a striking acceleration in the onset of mammary tumor formation by 13 weeks, representing approximately one-third of the life span of these animals, accompanied by an increase in the percent of animals with tumors at a defined end point. Because the effects of MAT and HANs on tumor development is similar, it is likely that the cellular changes induced by MAT that result in the development of HANs are the same changes that accelerate neu-induced tumor formation.

The mechanism underlying the tumor-enhancing property of MAT expression in MMTV-neu mice was addressed in this study. We hypothesized that MAT activity could result in an increase in the availability of soluble ErbB receptor ligands, through either cleavage of membrane precursors or release of ligand from matrix components. We tested the role of Neu-related signal transduction in MAT-accelerated tumorogenesis by comparing the levels, the ability to heterodimerize, and the activation of the ErbB receptors, as determined by phosphorylation. We observed no obvious differences in these parameters in MMTV-neu versus MMTV-MAT/neu tumors or in mammary glands that were prone to develop tumors. Rather, both groups of mammary tumors contain deletions within the neu transgene at approximately the same frequency. Although we cannot rule out the possibility that differences in ErbB signaling are too subtle to detect with these assays or that aberrant signaling is restricted to specific states that were not tested in these studies, these data imply that the presence of MAT does not alter signaling through the ErbB family of receptors and that the effects on HANs and neu-induced tumor acceleration are independent of ErbB signaling.

The mechanism by which MAT induces HAN formation and acceleration in the onset of mammary tumor formation by 13 weeks, representing approximately one-third of the life span of these animals, accompanied by an increase in the percent of animals with tumors at a defined end point. Because the effects of MAT and HANs on tumor development is similar, it is likely that the cellular changes induced by MAT that result in the development of HANs are the same changes that accelerate neu-induced tumor formation.

Fig. 5. Altered neu transcripts are expressed in MMTV-MAT/neu mammary tumors. Total RNA or poly(A)+ selected RNA isolated from MMTV-MAT/neu mammary glands, and mammary tumors were analyzed by RNase protection assay using an antisense rat neu cDNA riboprobe. The protected wild-type neu transcript was 60 nucleotides long, and protected fragments corresponding to altered transcripts (samples 9, 12, 13, and 14) are indicated with arrows. A 124-nucleotide mouse phosphoglycerate kinase (PGK-1) riboprobe was used to control for equal loading of sample RNA onto the polyacrylamide gel. Positions of DNA markers are shown on the left.

5504
erates tumor onset is not clear. The expression of MAT could induce hyperplastic lesions and accelerate the occurrence of neu deletions through an effect on cellular turnover by altering the rate of cell proliferation and/or apoptosis. An increase in the number of epithelial cells in the population would increase the chance of a random mutation, which could generate a selective advantage for that cell and initiate the carcinogenic process. An increase in both proliferation and apoptotic indices, accompanied by morphological alterations, was observed in transgenic mice in which the MMP STR-1 was targeted to the mammary epithelial cells (34–36). However, we observed no morphological changes in the ductal structures of MMTV-MAT mice, nor could we detect alterations in cellular proliferation as measured by proliferating cell nuclear antigen labeling or programmed cell death as measured by the terminal deoxynucleotidyl transferase–mediated nick end labeling assay (20). It is possible that the assays used were not sensitive enough to detect slight alterations in the proliferative or apoptotic indices of the normal or malignant mammary epithelial cells. Alternatively, the effects of MAT may be specific for a distinct stage of mammary physiology, perhaps explaining why premalignant HANs are observed in the glands of mice that have undergone several pregnancies. The effect has similarities to those described for tumor promoters in that by itself, MAT expression induces hyperplasia but not malignant tumors and acts in conjunction with an initiating event (deletions in the neu transgene) to accelerate tumor development. Induction of cellular proliferation is a necessary component of tumor promotion, but not all tumor promoters show a generalized hyperplastic response (Ref. 37, for example). An analysis of the cellular and molecular alterations in MMTV-MAT mammary epithelium and HANs may shed light on the mechanisms underlying this promotional activity.

MMTV-neu transgenic mice provide a reasonable model of human breast tumor progression in that they spontaneously develop adenocarcinomas with metastatic potential. The introduction of MMTV-driven MAT into these tumors in bigenic mice resulted in a small but not statistically significant increase in the percentage of mice with lung metastases. The lack of statistical significance may be a result of the relatively high rate of metastasis observed in the MMTV-neu control animals in this study. However, we also noted that the MAT transgene is expressed only sporadically in the periphery of advanced lesions (Fig. 2D), perhaps due to the loss of differentiation characteristics in these adenocarcinomas and reduction in expression of the MMP promoter. With these caveats, our studies provide no evidence to suggest that MAT plays a role in the metastatic spread of mammary adenocarcinomas. It is not clear whether the effect of MAT on tumorigenesis is related to a specific property of the MAT enzyme or is a result of the unusual expression pattern of this MMP, i.e., its expression in the epithelial component of early lesions. Recent studies in which the stromal MMPs STR-1 and STR-3 were expressed in normal or malignant mammary epithelial cells suggest it may be the latter. Transgenic mice expressing STR-1 under the control of the mammary epithelial cell–specific whey acidic protein promoter spontaneously develop malignant mammary adenocarcinomas (38). The difference in the extent of the tumorigenic phenotype of these mice compared to the MMTV-MAT mice (the development of malignant rather than premalignant lesions) may pertain to the differences in the promoter used, the MMP expressed, and/or the strain of the host mouse. The expression of STR-3 in human breast cancer cell lines does not increase the proliferation rate of the cells, or the metastatic capacity following injection into nude mice. However, cell lines expressing STR-3 develop tumors faster than do the parental cell lines that do not express STR-3 (39). Taken together, these results suggest that MMP activity in general enhances properties of mammary epithelial cells that allow them to establish tumors.

The appearance of HANs in the MMTV-MAT animals and the acceleration of MMTV-neu–induced tumors in MMTV-MAT/neu transgenic mice indicates that the expression of MAT in the mammary epithelium contributes to early-stage mammary tumorigenesis. Although the relationship between the HANs observed in mouse models and human breast pathologies is not clear, MAT has been observed in some benign (4) and noninvasive (6) breast lesions, as well as in apparently normal mammary epithelium (6, 9). Our results with this animal model system suggest that inhibition of MAT activity in individuals with an elevated risk for mammary carcinoma may provide a protective advantage and provide an incentive to pursue additional clinical and preclinical studies with synthetic MMP inhibitors to test the potential of this strategy in the prevention of malignant breast disease.

ACKNOWLEDGMENTS

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