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Antitumor Activity of the Novel Human Breast Cancer Growth Inhibitor, Mammary-derived Growth Inhibitor-related Gene, MRG

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Abstract

A novel human tumor growth inhibitor was identified by differential cDNA sequencing. The predicted amino acid sequence of this tumor-suppressing factor has a significant sequence homology to mouse mammamary-derived growth inhibitor and thus was named mammamary-derived growth inhibitor-related gene (MRG). MRG was found to be expressed in normal and benign human breast tissues but not in breast carcinomas. In situ hybridization analysis demonstrated a stage-specific MRG expression as follows. MRG was barely detectable in breast carcinomas, showed partial and weak expression in benign hyperplasia, but was expressed at a high level in normal breast epithelial cells. To determine if MRG can modulate in vivo growth of human breast cancers, we transfected a full-length MRG cDNA into MDA-MB-231 human breast cancer cells and studied the orthotopic growth of MRG transfectants versus control transfectants in the mammary fat pad of athymic nude mice. Overexpression of MRG in human breast cancer cells significantly suppressed cell proliferation in vitro and tumor growth in an orthotopic nude mouse model. These results suggest that MRG has tumor-suppressing activity, and the loss of MRG expression may be involved in the development and progression of breast cancer.

Introduction

Development of cancer and subsequent malignant progression are associated with multiple genetic changes that result in qualitative and quantitative alterations in individual gene expression (1). Many of these quantitative genetic changes manifest themselves as alterations in the cellular complement of novel transcribed mRNAs. Identification of these mRNAs, if sufficiently characterized, could provide clinically useful information for patient management while enhancing our understanding of cancer pathogenesis. The outcome of cancer growth can be determined by the balance between growth factors and oncogenes and growth inhibitors and tumor suppressor genes in the tumor environment. The list of growth inhibitors or tumor suppressor genes remains slim in comparison to the hundred-odd growth factors or oncogenes presently recognized.

Breast cancer evolves by clonal selection of cells that acquire multiple genetic changes. One proposed model for such accumulation suggests that breast cancer, like colon cancer (2), develops through defined morphologically distinguishable stages beginning with benign hyperplasia, progresses to atypical hyperplasia, which leads to carcinoma in situ, and finally to an invasive carcinoma (3). It has been proposed that the loss of tumor suppressors or growth inhibitors that locally signal growth cessation and stimulate differentiation of the developing mammary epithelium plays a critical role in this sequential development of breast cancer (4, 5). Presently, only a few naturally occurring growth inhibitors have been identified. These include transforming growth factor β (6, 7), interferons (8), tumor necrosis factor (9), and MDGI (10–13) for mammary epithelial cells. Their roles in the onset and progression of both hereditary and sporadic breast cancer are not defined.

We have used a differential cDNA sequencing approach (14) to search for potential tumor suppressor genes, the expressions of which are down-regulated or lost during breast cancer onset and progression. Differential cDNA sequencing is an EST sequencing-based method of differential expression cloning. Since the introduction of the EST sequencing approach, many novel human genes have been discovered (15, 16). The advantage of this methodology, compared to isolation and sequencing of individual cDNAs, is that a large number of sequences can be "catalogued" with small amounts of sequencing data. Therefore, unlike differential display and subtractive hybridization which require the relative time- and labor-intensive steps of subcloning, library screening, and cDNA sequencing of individual genes (17, 18), the creation of EST libraries is a rapid method used to identify or "tag" sequences that are expressed in specific tissues (15, 19). A novel growth inhibitor, MRG, with sequence homology to MDGI for breast cancer was identified as down-regulated in breast cancer compared with normal breast.

MDGI is a mammary epithelial cell growth inhibitor and differentiation factor initially identified and purified from Ehrlich ascites mammary carcinoma cells (10) and then from the lactating bovine mammary gland (11, 12) and cows' milk (13). MDGI revealed no homology to any other known growth inhibitors (20); rather, MDGI belongs to a multigene family of FABPs (21). Thus far, only bovine (11, 12) and mouse (22, 23) MDGI were identified and characterized. In this study, the expression of MRG in human breast and its antitumor activity are characterized. Our results suggest that MRG is a tumor suppressor gene, and its loss of expression in breast carcinoma may contribute to breast cancer onset and progression.

Materials and Methods

Reagents. Restriction enzymes, T7 polymerase, random primer DNA labeling kit, and digoxigenin-labeled nucleotides were obtained from Boehringer Mannheim (Indianapolis, IN). [32P]dATP was purchased from Amersham Corp.

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3 The abbreviations used are: MDGI, mammamary-derived growth inhibitor; EST, expressed sequence tag; MRG, MDGI-related gene; FABP, fatty acid-binding protein; H-FABP, heart-derived FABP; A-FABP, adipocyte-derived FABP; DCIS, ductal carcinoma in situ; MVC, microvesSEL count.

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Molecular Cloning of MRG Full-length cDNA Sequence. We used EST-based automatic differential cDNA sequence analysis (14) to search for new genes differentially expressed in breast cancer versus normal breast. Briefly, a data base containing approximately one million human partial cDNA sequences (ESTs) has been established in a collaborative effort between the Institute for Genomic Research and Human Genome Science, Inc., using a high throughput automated DNA sequence analysis of randomly selected human cDNA clones (16). RNAs from a breast carcinoma and patient-matched normal breast were isolated and subjected to preparation of cDNA libraries. EST automated DNA sequence analysis was performed on randomly selected cDNA clones. The ESTs with overlapping sequences were grouped into unique EST groups. Each EST group may represent a gene or a family of sequence-related genes. Each unique EST group was analyzed for its relative expression by examining the number of expressed individual ESTs in the libraries of normal versus cancerous breast tissue. The numbers of EST hits in the libraries reflect the relative expression or mRNA transcript copy numbers of the EST. There were more than 5000 EST groups that were analyzed for quantitative comparison of EST hits in the pairs of cDNA libraries from normal breast versus breast cancer by examining the expression of individual EST sequences. Among several differentially expressed EST groups (14), one EST with full-length cDNA sequence from a normal breast library, which has sequence homology to MDGI, was completely sequenced and named as MRG.

In Situ Hybridization. In situ hybridization was carried out as we described previously (14). Briefly, deparaffinized and acid-deproteinized sections (5-μm thick) were treated with protease K, prehybridized, and hybridized overnight with digoxigenin-labeled antisense transcripts from an MRG cDNA insert. The MRG antisense probe is a 731-bp, full-length fragment. The probe was generated by EcoRI digestion of MRG cDNA plasmid and followed by T7 polymerase. Hybridization was followed by RNase treatment and three stringent washes. Sections were incubated with mouse anti-digoxigenin antibodies (Boehringer Mannheim), followed by incubation with biotin-conjugated secondary rabbit antimouse antibodies (DAKO). The colorimetric detection was performed by using a standard indirect streptavidin-biotin immunoreaction method by using the Universal LSAB kit (DAKO) according to the manufacturer's instructions. There were some variations in staining intensity for MRG expression among the specimens. Because the colorimetric in situ hybridization is not quantitative, the tissue samples were classified into three classes: negative (−), strongly positive (+), and weakly positive (+). The negative cases were confirmed with at least two independent experiments. All stainings were reviewed by two pathologists.

Transfection. The full-length MRG cDNA was inserted into a pC1-neo mammalian expression vector. The resulting vector was transfected into MDA-MB-231 cells as we described previously (24). Subsequent to transfection, G418 selection, and cloning by limiting dilution, several subclones of MDA-MB-231 cells were obtained. These G418-resistant clones were expanded into individual cell lines and used as a source for RNA analysis. Clones were initially screened by in situ hybridization with a specific MRG antisense probe, and the positive clones were subjected to Northern blot analysis.

Northern Blot Analysis. Detection of MRG mRNA expression was analyzed by Northern blot as we described previously (24).

In Vitro Assay for Cell Growth. Exponentially growing cultures of different MDA-MB-231 clones were detached with trypsin, and the trypsin was neutralized with DMEM-10% serum. Cells were counted, dilute, and seeded in triplicate at 3000 cells/well (24-well plate) in 1 ml of DMEM-5% serum. Cell growth was measured using CellTiter 96 Aqueous Nonradioactive cell proliferation assay kit (Promega Corp.).

Tumor Growth in Athymic Nude Mice. A nude mouse tumorigenic assay was performed as we described previously (25). Briefly, cells were grown to 80–90% of confluence in 150-cm² dishes and were harvested by incubation with 5 mm EDTA in PBS. The EDTA was neutralized with medium containing serum. The cells were washed twice with serum-free medium, counted, and resuspended in serum-free DMEM at a concentration of 2.7 × 10⁶ cells/ml. Approximately 0.4 × 10⁶ cells (0.15 ml) were injected into a female athymic nude mouse 5–6 weeks of age (Frederick Cancer Research and Development Center, Frederick, MD). Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples. The animals were ear tagged. Primary tumor growth was assessed by measuring the volume of each tumor at weekly intervals. Tumor size was determined at intervals by three-dimensional measurements (mm) using a caliper. Only measurable tumors were used to calculate the tumor volume for each tumor cell clone at each time point. Animals were sacrificed between 32 and 40 days after injection, when the largest tumors reached about 15 mm in diameter.

Statistical Analysis. Values were expressed as means ± SE. Where appropriate, comparisons were made using the two-tailed Student's t test.

Results

Molecular Cloning of MRG cDNA. We generated cDNA libraries from a breast cancer biopsy specimen and a normal breast and analyzed these libraries by EST-based differential cDNA sequencing. ESTs were sequenced and grouped to different groups based on the sequence homology. Each unique EST group was first analyzed for relative expression in the cDNA sequence of normal breast versus breast cancer and then subjected to tissue-specific expression by examining tissue origins of individual EST sequences against approximately one million ESTs derived from a variety of different tissue types. As demonstrated previously (14), we identified three classes of EST groups that were differentially expressed in normal breast versus breast cancer: (a) genes that are more abundant in breast cancer than in normal breast; (b) genes that are more abundant in normal breast than in breast cancer; and (c) genes that are selectively expressed in the breast relative to other tissue types. Within the second class, automated screening revealed a group of 25 ESTs encoding a novel gene with homology to mouse MDGI (greater than 30% homology). Of the 25 distinctive MDGI-related EST clones, 4 ESTs were derived from the normal breast library; the rest were derived from a fetal brain library. No ESTs were derived from the breast cancer library. After sequencing of these cDNA fragments, one clone containing a start codon (ATG) and an open reading frame was isolated and characterized.

The nucleotide sequence determined from this clone and the predicted corresponding amino acid sequences are shown in Fig. 1. The full-length cDNA sequence contains 731 bp with a 396-bp open reading frame. The amino acid sequence contains 260 amino acids. The open reading frame begins with an ATG and ends with a TAA stop codon. The open reading frame extends from the initiation A₇₆TG codon to TAA₇₇₄ stop codon.

![Fig. 1. MRG cDNA sequence.](image-url)
The amino acid sequence of MRG was aligned with those of bovine MDGI, mouse MDGI, human H-FABP, and human A-FABP. The available amino acid sequence of bovine and mouse MDGI and human H-FABP and A-FABP were obtained from the SwissProt data base and aligned with the MRG deduced sequence using the clustal method of the MegAlign Program from the DNASTAR software package. Conserved amino acids are shaded.

A-FABP. The available amino acid sequence of bovine and human MRG with mouse and bovine MDGI and human FABP. The available amino acid sequence of bovine and mouse MDGI and human H-FABP and A-FABP were obtained from the SwissProt database and aligned with the MRG deduced sequence using the clustal method of the MegAlign Program from the DNASTAR software package. Conserved amino acids are shaded.

Expression of MRG in Human Breast Epithelial Cells. We speculate that MRG is a potential tumor suppressor gene or growth inhibitor for mammary gland because: (a) the differential cDNA sequencing revealed MRG expression in the normal breast library but not in the breast cancer library; and (b) it has a sequence homology to MDGI, a bovine- and mouse-derived growth inhibitor for mammary epithelial cells described previously. In an attempt to evaluate the potential biological significance of MRG on human breast cancer development and progression, we first studied MRG gene expression in human biopsy samples from breast carcinomas and normal breast tissues. Northern blot analysis (Fig. 3A) allowed the detection of MRG expression in all four normal breast reduction mammoplasty specimens but not in two malignant breast cancer samples. The presence of MRG in normal breast and the loss of MRG expression in breast carcinomas suggest a possible role of down-regulation of MRG in the development of breast cancer. The expression of MRG was also investigated in some human breast cancer cell lines (Fig. 3B). No expression of MRG was detected in any of the tested human breast cancer cell lines except T47D cells.

To localize the cellular source of MRG expression and to further assess the biological relevance of the loss of MRG expression in breast cancers, we next performed in situ hybridization on the fixed sections from a variety of different human breast specimens. In these experiments, we examined two aspects of MRG expression, including the tissue localization (stromal versus epithelial) and the correlation of the loss of MRG expression and breast cancer malignant phenotype. Fig. 4 shows a representative in situ hybridization for MRG. We found a strongly positive MRG hybridization in epithelial cells of normal mammary glands (Fig. 4, A and B) and benign fibroadenomas (Fig. 4C). The expression of MRG mRNA was detectable in the epithelial cells in all five reduction mammoplasty specimens and in five benign fibroadenomas. In contrast, expression of MRG was absent in 9 of 10 cases of infiltrating breast carcinomas and in 10 of 12 DCIS. Representative negative stainings of MRG in DCIS neoplastic epithelial cells (Fig. 4E) and in an infiltrating breast carcinoma (Fig. 4F) are presented. These in situ hybridization results are consistent with the Northern blot analysis that showed MRG expression in normal breast but not in breast carcinomas.

It is interesting that although a strong MRG signal was easily detected in the breast epithelial cells of normal glands and benign fibroadenomas, the benign breast hyperplasia showed a different MRG expression pattern. Among 10 benign hyperplasias, 4 specimens were negative and 6 specimens were lightly stained for MRG expression. As illustrated in Fig. 4D, the intensity of MRG staining in a benign hyperplasia was greatly reduced compared to the normal mammary tissue localization (stromal versus epithelial) and the correlation of the loss of MRG expression and breast cancer malignant phenotype. Fig. 4 shows a representative in situ hybridization for MRG. We found a strongly positive MRG hybridization in epithelial cells of normal mammary glands (Fig. 4, A and B) and benign fibroadenomas (Fig. 4C). The expression of MRG mRNA was detectable in the epithelial cells in all five reduction mammoplasty specimens and in five benign fibroadenomas. In contrast, expression of MRG was absent in 9 of 10 cases of infiltrating breast carcinomas and in 10 of 12 DCIS. Representative negative stainings of MRG in DCIS neoplastic epithelial cells (Fig. 4E) and in an infiltrating breast carcinoma (Fig. 4F) are presented. These in situ hybridization results are consistent with the Northern blot analysis that showed MRG expression in normal breast but not in breast carcinomas.

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glands (Fig. 4, A and B) or benign fibroadenoma (Fig. 4C). The decreased MRG expressions in hyperplasia and the loss of MRG expression in breast carcinomas suggest that expression of MRG is stage specific in the breast: down-regulated during the proliferative stage and lost in breast cancer.

**Tissue Expression.** Tissue-specific transcription of MRG was examined by Northern blot on 2 μg of poly(A) RNAs from various human adult tissues (Fig. 5). As expected, the Northern blot showed that MRG was abundantly expressed as a 1.1-kb transcript in the brain, in which 21 MDGI-related ESTs were identified, and in heart and skeletal muscle, which are rich sources for H-FABP. A 2.2-kb transcript with much lower accumulation in relative intensity was also detected in the heart, skeletal muscle, and pancreas. By contrast, no MRG mRNAs were present in other specimens analyzed, such as placenta, lung, liver, kidney, spleen, thymus, prostate, testis, uterus, colon, and small intestine.
above tissues were purchased from Clontech. Using a full-length cDNA hybridization probe, a high abundance of 1.1-kb transcripts was detected in heart, brain, and skeletal muscle. Northern blots containing approximately 2 μg of poly(A) RNA per lane from each of the muscle. Weak 2.2-kb transcripts were also detected in heart, skeletal muscle, and pancreas. In contrast, no endogenous MRG transcripts were detected in all control clones and parental cells. Lane I, parental MDA-MB-231 cells; Lane 2, neo-231-I clone; Lane 3, and two MRG-transfected clones. Strong MRG transcripts were detected in MRG-positive clones. In the parental MDA-MB-231, neo-231-I, and neo-231-2 cells. The same breast cancer cells transfected with MRG, however, were significantly inhibited in their tumor growth; and either no or a low level of tumor necrosis was observed. The mean volume of MRG-231-6 and MRG-231-10 tumors was only 21% of that in parental MDA-MB-231 tumors, 24% of that in neo-231-1 tumors, and 18% of that in neo-231-2 tumors, respectively (P < 0.005 by Student's t test). Fig. 8 shows growth kinetics of one representative experiment from parental MDA-MB-231, neo-231-1, neo-231-2, MRG-231-6, and MRG-231-10 tumors. A slow growth phase of 25 days, tumors from both parental MDA-MB-231 cells and neo MDA-MB-231 clones increased in volume at an exponential rate. In contrast, the growths of

Transfection and Selection of MRG-positive Clones. Because we demonstrated a loss of MRG expression in breast cancers compared with normal or benign breasts, we next asked if we could suppress breast cancer growth by overexpression of the MRG gene in breast cancer cells. We selected the MDA-MB-231 cell line as the recipient for MRG-mediated gene transfection because of: (a) its lack of detectable MRG transcript; and (b) its more aggressive and highly tumorigenic behavior in nude mice. Cells were transfected with a plasmid vector containing a neomycin resistance gene (neo-231 clones). Both selected MRG-231 clones MRG-231-6 and MRG-231-10 expressed MRG mRNA transcripts. In contrast, none of the parental MDA-MB-231, neo-231-1, and neo-231-2 cells produced any detectable MRG transcripts.

Effect of MRG Transfection on Cell Growth. The growth curve of MRG-231 cells was compared to that of MRG-negative MDA-MB-231 and neo-231-1 cells in a monolayer culture (Fig. 7). Cell growth was inhibited approximately 65–75% (P < 0.001 by Student's t test) in the MRG-231-6 and MRG-231-10 cells compared to the parental MDA-MB-231 and neo-231-1 cells. There was no significant difference in the growth pattern between parental MDA-MB-231 cells and neo-231-1 cells.

Suppression of Tumorigenicity by MRG Transfection. To study the effect of MRG expression on tumorigenicity, the tumor growths of MRG-231 clones were determined in comparison with parental MDA-MB-231 cells and neo-231 clones in an orthotopic nude mouse model. Two independent experiments were done to confirm reproducibility, and the data are shown in Table 1. After a lag phase of approximately 10 days, mice given implants of both MRG-positive and MRG-negative cells developed tumors. There was no significant difference in tumor incidence among the groups. Starting at about 30 days after inoculation, a high level of tumor necrosis was observed in tumors derived from MDA-MB-231, neo-231-1, and neo-231-2 cells. The same breast cancer cells transfected with MRG, however, were significantly inhibited in their tumor growth; and either no or a low level of tumor necrosis was observed. The mean volume of MRG-231-6 and MRG-231-10 tumors was only 21% of that in parental MDA-MB-231 tumors, 24% of that in neo-231-1 tumors, and 18% of that in neo-231-2 tumors, respectively (P < 0.005 by Student's t test). Fig. 8 shows growth kinetics of one representative experiment from parental MDA-MB-231, neo-231-1, neo-231-2, MRG-231-6, and MRG-231-10 tumors. After a slow growth phase of 25 days, tumors from both parental MDA-MB-231 cells and neo MDA-MB-231 clones increased in volume at an exponential rate. In contrast, the growths of

![Fig. 5. The expression of MRG gene in a variety of normal human adult tissues. Two Northern blots containing approximately 2 μg of poly(A) RNA per line from each of the above tissues were purchased from Clontech. Using a full-length cDNA hybridization probe, a high abundance of 1.1-kb transcripts was detected in heart, brain, and skeletal muscle. Weak 2.2-kb transcripts were also detected in heart, skeletal muscle, and pancreas.](image)

![Fig. 6. Northern blot analysis of MRG transfection of MDA-MB-231 cells. Total RNAs were isolated from parental MDA-MB-231 cells, two control pCI-neo-transfected clones, and two MRG-transfected clones. Strong MRG transcripts were detected in MRG-positive clones. In contrast, no endogenous MRG transcripts were detected in all control clones and parental cells. Lane 1, parental MDA-MB-231 cells; Lane 2, neo-231-1 clone; Lane 3, neo-231-2 clone; Lane 4, MRG-231-6 clone; Lane 5, MRG-231-10 clone. The integrity of the RNAs and loading control was ascertained by visualization of the 18S rRNA bands in the stained gel (data not shown).](image)

![Fig. 7. Effect of MRG overexpression on growth. The growth rates of MRG-positive clones were compared to that of MRG-negative clones in monolayer culture as described in "Materials and Methods." The number represents the mean of three cultures; bars, SE.](image)

![Table 1 Effects of MRG expression on tumor incidences and sizes of MDA-MB-231 cells](image)
The expression of mouse MDGI in mammary epithelial cells is hormone-regulated and maximally expressed in the terminally differentiating lobuloalveolar epithelial cells of differentiated glands (26). These findings suggest that, in the nude mouse orthotopic model, breast cancer angiogenesis is highly related to primary tumor size. The larger primary tumors usually have more angiogenic activity and more MVCs. Because MRG has been demonstrated to inhibit breast cancer growth (31), within the same context, we demonstrated that overexpression of MRG in MDA-MB-231 human breast cancer cells significantly inhibited their tumor growth by 4-6-fold as compared to MRG-negative control tumors in nude mice. This MRG-mediated in vivo tumor growth inhibition is consistent with its in vitro inhibition of cell growth.

Angiogenic regulatory factors have been found to modulate the growth of human breast cancers in several orthotopic xenograft models. Recent studies suggest that MCVs of paraffin sections from human breast cancers can be used as a measure of tumor angiogenesis, and that these MCV measurements are independent markers of prognosis (32, 33). We have recently demonstrated that transfection of MDA-MB-435 cells with an angiogenic factor, scatter factor, increased tumor growth, MCV values, and angiogenesis (34). On the other hand, overexpression of tissue inhibitor of metalloproteinase 4 in human breast cancer cells results in significant inhibition of tumor growth, metastasis, and MCV values (35). As we described previously (34, 35), we performed MVC analysis in an attempt to investigate if inhibition of tumor growth by MRG is mediated by the inhibition of angiogenesis. We found that MCV values were significantly decreased in MRG-positive tumors as compared to that in the MRG-negative tumors (data not shown). Thus, using MCV values as the criterion, MRG inhibited tumor angiogenesis. However, we are aware that, in the nude mouse orthotopic model, breast cancer angiogenesis is highly related to primary tumor size. The bigger primary tumor usually has more angiogenic activity and more MCVs. Because MRG transfectants were significantly inhibited in their tumor growth compared with MRG-negative clones, it is expected that MVC values should be lower in MRG-positive clones than those of MRG-negative clones, and the lower MVC values may reflect only the smaller tumors.

It is interesting that MRG mRNA was detected in T47D hormone-responsive human breast cancer cells by Northern blot, although no mRNAs were detectable in other tested human breast cancer cell lines. The expression of mouse MDGI in mammary epithelial cells is hormonally regulated and maximally expressed in the terminally differentiated glandular epithelium. It will be interesting to investigate whether the down-regulation of MRG in a benign hyperplasia may indicate a malignant progression to an atypical hyperplasia and subsequent development of a breast carcinoma. If MRG expression can provide some prognostic information on distinguishing a benign hyperplasia that is not likely to have malignant progression and an atypical hyperplasia that is likely to progress to a carcinoma, this will help to direct the treatment strategies and to reduce the development of breast cancer.

MRG revealed no sequence homology to any of the hitherto known growth inhibitors except partial homology to mouse and bovine MDGI. Until now, only bovine (11, 12) and mouse MDGI (22, 23) have been identified and characterized. Studies of mouse and bovine MDGI suggest several potential functions of MDGI on the growth and differentiation of the mammary gland. Recombinant and native mouse MDGI specifically inhibit the growth of normal mouse mammary epithelial cells and promote morphological differentiation and milk protein synthesis (26). Selective inhibition of endogenous MDGI expression in mouse mammary epithelial cells by use of antisense oligonucleotides suppresses alveolar budding and impairs β-casein synthesis in organ cultures (26). Increasing amounts of MDGI mRNA were detected in the epithelium of developing lobules and in terminal parts of ducts and lobuloalveolar epithelial cells of differentiated glands (27). These data suggest a local role of MDGI as a growth inhibitor in mediating or complementing hormonal action during differentiation (28, 29). In this regard, MDGI is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells (30), which may be involved in the protective effect of early parity on subsequent breast cancer incidence. Previously, bovine MDGI has been demonstrated to inhibit breast cancer growth (31). Within the same context, we demonstrated that overexpression of MRG in MDA-MB-231 human breast cancer cells significantly inhibited their tumor growth by 4-6-fold as compared to MRG-negative control tumors in nude mice. This MRG-mediated in vivo tumor growth inhibition is consistent with its in vitro inhibition of cell growth.

Many breast tumors go through a series of events from the time of initial detection to the formation of the lethal metastatic stage. Peptides that locally signal growth cessation and stimulate differentiation of the developing epithelium are very important for mammary gland development and preventing cancer formation. Identification of these peptides, if sufficiently characterized, may yield molecular markers that may be useful in the diagnosis and treatment of breast cancer. The differential cDNA sequencing method (14) was applied to the isolation of transcriptionally regulated genes involved in the development of human breast cancer. We reported here a novel MDGI-related growth inhibitor, MRG, that was expressed in normal and benign breast epithelial cells but was rarely expressed or absent in malignant breast epithelial cells. Overexpression of MRG in MDA-MB-231 human breast cancer cells inhibited cell growth. When injected orthotopically into nude mice, MRG transfectants were significantly inhibited in their tumor growth as compared with controls.

Using in situ hybridization analysis, we have demonstrated the expression of MRG transcripts in the epithelial cells of normal and benign breast but rarely in the neoplastic epithelial cells of breast carcinomas. The high expression (10 of 10) of MRG gene in the epithelial cells of normal breasts and benign fibroadenomas compared to extremely low expression (3 of 22) in the malignant epithelial cells of breast carcinomas suggests that the loss or down-regulation of MRG expression is associated with breast cancer development. This implication is further supported by the detection of partial and weak MRG expression in benign hyperplasias. Only 6 of 10 benign hyperplasias stained for MRG expression. In addition, the decreased MRG is evident in these rapidly proliferating nonmalignant breast lesions (Fig. 4D). It is likely that the down-regulation of MRG expression is the consequence of cellular proliferation.

It will be interesting to investigate whether the down-regulation of MRG in a benign hyperplasia may indicate a malignant progression to an atypical hyperplasia and subsequent development of a breast carcinoma. If MRG expression can provide some prognostic information on distinguishing a benign hyperplasia that is not likely to have malignant progression and an atypical hyperplasia that is likely to progress to a carcinoma, this will help to direct the treatment strategies and to reduce the development of breast cancer.
differentiated state found just prior to lactation (27). In addition, mouse MDGI in mouse mammary epithelial cells was increased during the functional differentiation induced by progesterone- and prolactin (28, 29). Because T47D cells, unlike MCF-7 cells, are primarily progestin-responsive cells because of the higher differentiated state found just prior to lactation (27). In addition, described previously for MDGI (26) is a mix of H-FABP and A-hormonal regulation and contribute to the progestational regulation of mones and prolactin (28, 29). Because T47D cells, unlike MCF-7 mouse MDOI in mouse mammary epithelial cells was increased based on the complete cDNA and protein homologies. These results suggest that the functions of MDGI described previously are exerted by FABP (38). Unlike MDGI which has almost identical sequence to H-FABP, MRG has relatively low sequence identity to human H-FABP (66%). In addition, in contrast to MDGI described previously, the growth-inhibitory activity of a partially purified recombinant MRG protein from a baculovirus expression system is not limited to mammary cells. The growth of human prostate cancer and cervical cancer cells were also inhibited by MRG protein. These results suggest that MRG may represent a MDGI-related but different class of growth inhibitor. Thus, along with interferons, transforming growth factor β, tumor necrosis factor, and MDGI, MRG is one of the few naturally occurring growth inhibitors for mammary epithelium.

The magnitude of the tumor-suppressing activity of MRG is comparable to that observed previously for Rb and p53 (40). The loss of MRG expression in malignant breast cancers and the inhibition of breast tumor growth by reexpression of MRG suggest that MRG is one of the growth inhibitors that locally signals growth cessation of the mammary gland. In fact, the human homologue of bovine MDGI has been mapped to chromosome 1p32–35 (31), a locus shown previously to exhibit frequent loss of heterozygosity in human sporadic breast cancer (41, 42). Therefore, the loss or down-regulation of MRG or its receptor may lead to abnormal growth and the development of breast cancer.

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References

MRG IN HUMAN BREAST CANCER


Antitumor Activity of the Novel Human Breast Cancer Growth Inhibitor, Mammary-derived Growth Inhibitor-related Gene, MRG


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