Induction of Mammary Differentiation by Mammary-derived Growth Inhibitor-related Gene That Interacts with an ω-3 Fatty Acid on Growth Inhibition of Breast Cancer Cells

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ABSTRACT

We previously identified and characterized a novel tumor growth inhibitor and a fatty acid-binding protein in human mammary gland and named it the mammary-derived growth inhibitor-related gene (MRG). Here, the effects of MRG on mammary gland differentiation and its interaction with ω-3 polyunsaturated fatty acids (ω-3 PUFAs) on growth inhibition were investigated. MRG protein expression was associated with human mammary gland differentiation, with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland. Overexpression of MRG in human breast cancer cells induced differentiation with changes in cellular morphology and a significant increase in the production of lipid droplets. Treatment of mouse mammary gland in organ culture with MRG protein resulted in a differentiated morphology and stimulation of β-casein expression. Treatment of human breast cancer cells with the ω-3 PUFA docosahexaenoic acid resulted in a differential growth inhibition proportional to their MRG expression. MRG-transfected cells or MRG protein treated cells were much more sensitive to docosahexaenoic acid-induced growth inhibition than MRG-negative or untreated control cells. Our results suggest that MRG is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells and may play a major role in ω-3 PUFA-mediated tumor suppression.

INTRODUCTION

MRG has been cloned in normal human mammary gland by differential cDNA sequencing aimed at the identification of growth inhibitory factors of the normal mammary gland (1). The sequence of MRG was found to be identical to the recently identified human B-FABP (Ref. 2). FABPs constitute a well-established family of cytoplasmic hydrophobic ligand-binding proteins and are thought to be involved in lipid metabolism by binding and transporting long-chain fatty acids intracellularly. However, other studies have implicated different roles for FABPs in cell signaling, growth inhibition, and differentiation (3–6). In particular, H-FABP, also known as MDGI, is abundantly expressed in differentiated lactating mammary gland and has been shown to inhibit growth of breast cancer cells (7–9). Among several subtypes of FABPs, only MRG/B-FABP and MDGI, are abundantly expressed in differentiated alveolar mammary epithelial cells from the lactating gland. MDGI, is abundantly expressed in differentiated lactating mammary gland and has been shown to inhibit growth of breast cancer cells (10). Among several subtypes of FABPs, only MRG/B-FABP and MDGI, have tumor-suppressing activity (2). Among several subtypes of FABPs, only MRG/B-FABP and MDGI, have tumor-suppressing activity (2). Among several subtypes of FABPs, only MRG/B-FABP and MDGI, have tumor-suppressing activity (2). Among several subtypes of FABPs, only MRG/B-FABP and MDGI, have tumor-suppressing activity (2).

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We previously identified and characterized a novel tumor growth inhibitor and a fatty acid-binding protein in human mammary gland and named it the mammary-derived growth inhibitor-related gene (MRG). Here, the effects of MRG on mammary gland differentiation and its interaction with ω-3 polyunsaturated fatty acids (ω-3 PUFAs) on growth inhibition were investigated. MRG protein expression was associated with human mammary gland differentiation, with the highest expression observed in the differentiated alveolar mammary epithelial cells from the lactating gland. Overexpression of MRG in human breast cancer cells induced differentiation with changes in cellular morphology and a significant increase in the production of lipid droplets. Treatment of mouse mammary gland in organ culture with MRG protein resulted in a differentiated morphology and stimulation of β-casein expression. Treatment of human breast cancer cells with the ω-3 PUFA docosahexaenoic acid resulted in a differential growth inhibition proportional to their MRG expression. MRG-transfected cells or MRG protein treated cells were much more sensitive to docosahexaenoic acid-induced growth inhibition than MRG-negative or untreated control cells. Our results suggest that MRG is a candidate mediator of the differentiating effect of pregnancy on breast epithelial cells and may play a major role in ω-3 PUFA-mediated tumor suppression.

MATERIALS AND METHODS

Cell Culture. Human breast cancer cell lines MDA-MB-231, MDA-MB-436, and MDA-MB-468 were maintained in DMEM containing 5% FCS.

Preparation of Anti-MRG Antibody. A peptide sequence corresponding to amino acids 43–57 (1) was chosen for developing of the antibody because of its unique sequence for MRG. The peptide synthesis, purification, conjugation, and immunization of rabbits were conducted as we described previously (24). For final purification, a MRG peptide affinity column was made by conjugating 20 mg of MRG peptide to 5 ml of Aminolink resin (Pierce Chemical Co.), using sodium cyanoborohydride (Sigma).

Immunohistochemical Staining. As described previously (1, 25), deparaffinized, rehydrated, and acid-treated human breast sections (5 μm thick) were treated with H2O2 and trypsin, and blocked with normal goat serum. Sections were incubated with a specific anti-MRG monoclonal antibody (1 μg/ml) at 4°C overnight, followed by incubation with biotin-conjugated secondary antirabbit antibodies (DAKO). The colorimetric detection was performed using a standard indirect streptavidin-biotin immunoreaction method with DAKO’s Universal LSAB Kit according to the manufacturer’s instructions. There were some variations in staining intensity for MRG expression among the specimens. The negative cases were confirmed with at least two independent experiments. All stainings were reviewed by two pathologists.

Preparation of MRGp. The full-length MRG was amplified using standard PCR techniques with primers corresponding to the 5’ and 3’ sequences of the gene (5’ primer, GGATCCCGTGAGGCTTTCTGT; 3’ primer, GGTACCCTAGGCCCCCAGACCTTT), using PfuTurbo DNA polymerase (Stratagene). The amplified fragment was gel-purified, and the DNA sequence was confirmed. As we described previously (24), a baculovirus expression vector, pA2-GP, was used to transform Sf9 cells. The purification of MRGp was performed as follows: (a) Medium supernatant, adjusted to pH 5.5, was first applied to tandem Poros HS/HQ columns (PerSeptive Biosystems) preequilibrated with 50 mM NaOAc (pH 5.5). (b) MRGp, collected in the flowthrough fraction, was adjusted to pH 8.0 and reapplied to the tandem Poros HS/HQ column preequilibrated with 20 mM Tris-HCl (pH 8.0). (c) MRGp, collected in the flowthrough fraction, was concentrated 50-fold, using a Filtron 3000 M cutoff tangential-flow system and then separated on a Superdex-75 size-exclusion column equilibrated with 10 mM NaOAc (pH 6.5). (d) Pooled

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MRGp fractions were applied to a hydroxyapatite column equilibrated with 10 mM NaOAc (pH 6.5); the weakly bound MRGp was eluted with 7.5 mM K$_2$HPO$_4$ (pH 6.8). (e) MRGp fractions were then separated on a Superdex-75 size-exclusion column equilibrated with 65 mM Na$_2$HPO$_4$, 100 mM NaCl (pH 7.2). MRGp fractions were pooled and found to be >98% pure by SDS-PAGE with an endotoxin level <0.5 endotoxin units/mg. Purified MRGp was identified as a single band at 18 kDa in the SDS-PAGE by silver staining. The protein was analyzed for glycosylation by determining the monosaccharide content in a purified preparation, and the N-linked sugar chains were confirmed.

Cell Morphology on Matrigel. Cell morphology was determined using Matrigel-coated wells. Briefly, 6-well culture plates were coated with growth factor-reduced Matrigel (Collaborative Research) at 0.5 ml/well. Cells were then cultured in the coated wells with DMEM containing 5% fetal bovine serum. The cell morphology was observed under the microscope after 4 days.

Detection of Cytoplasmic Lipids in Breast Cancer Cells. Lipid accumulation was detected by oil red O-isopropanol staining as described previously (26). The cells were cultured on either Matrigel-coated plates or regular uncoated plates. After 4 days, the cells were fixed by 10% formaldehyde and subjected to oil red O-isopropanol staining. Accumulated lipids in the cells were stained red, and nuclei were stained blue by hematoxylin. Three independent observers counted the positive cells, and each observer randomly counted three fields (×40). The numbers represent the average percentage of lipid accumulate cells from nine fields (×40).

Western Analysis. Western blot analysis was conducted as we described previously (24). Briefly, the blot was incubated with anti-MRG primary antibody (1:8000 dilution) overnight at 4°C, and then incubated with goat antirabbit IgG-horseradish peroxidase (1:6000 dilution) for 1 h, washed, and visualized by chemiluminescence.

Fig. 1. Analysis of MRG protein expression on human breast tissues by immunohistochemical staining. Sections in panels A and B were stained with H&E with no immunohistochemical staining. Sections in panels C–E were stained immunohistochemically, with brown indicating MRG protein expression in mammary epithelial cells. All sections in C–E were also counterstained lightly with hematoxylin for viewing non-MRG-stained cells. A, epithelial cells in normal nonlactating lobules from a normal breast reduction mammoplasty specimen (×40). B, epithelial cells in lactating lobules from a needle biopsy specimen (×40). The differentiated lactating mammary epithelial cells have much diluted cytoplasm containing large lipid-rich secretory vacuoles (arrow). C, epithelial cells from lactating lobules showed very strong MRG staining (×10). The specimen was from a 32-year-old lactating woman. The presence of vesicles containing milk protein (arrow) was noted. A serial slide from the same block was also incubated with nonimmunized control IgG, and no detectable background staining was observed at the same conditions as for the anti-MRG antibody. D, negative staining of normal lobular epithelial cells from a 23-year-old nulliparous woman with breast reduction mammoplasty (×10). E, negative staining of MRG in a highly infiltrating breast carcinoma (×10). A total of 23 clinical breast specimens were analyzed: 5 of 5 lactating samples were strongly positive; 10 of 10 infiltrating breast cancer samples were negative; 5 of 8 normal breast reduction mammoplasty samples were negative, and the remaining 3 normal breast samples were weakly positive.

**RESULTS**

Association of MRG Expression with Mammary Gland Lactation. In an attempt to evaluate the potential biological significance of MRG on the differentiation and lactation of the human mammary gland, we studied MRG protein expression in formalin-fixed, paraffin-embedded clinical human biopsy specimens from normal breast reduction mammoplasty specimens, lactating mammary glands, and malignant breast carcinomas.

Fig. 1 shows a representative immunohistochemical staining for MRG. The terminally differentiated lactating mammary gland is characterized by ducts branching into distended and large lipid-rich active secretory lobuloalveolar structures. An increase in cell volume as a
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Expression in MRG-231-10 and MRG-231-6 cells, two MRG-positive clones, but not in parental MDA-MB-231 and neo-231-1 MRG-negative cells.

It is well established that the extracellular matrix is required for normal functional differentiation of mammary epithelia. Striking changes in cell morphology were observed when MRG-231 cells were cultured in the Matrigel-coated dish. MRG-231-10 cells were aggregated to form spheroids on a reconstituted basement membrane gel (Fig. 3B), a typical differentiated phenotype for mammary epithelial cells (28). In contrast, neo-231-1 cells showed considerable heterogeneity in cell size, and many cells had “fibroblast-like” spreading morphology (Fig. 3C).

We examined whether MRG-induced morphological changes are consistent with differentiation. Because the maturation of breast cells is characterized by the presence of lipid droplets that are milk components, we examined lipid accumulation in MRG-231 cells compared with the control cells. Droplets containing neutral lipid were readily detectable in MRG-231-6 clones cultured in the uncoated culture plates; in contrast, no obvious lipid droplet could be observed in the neo-231-1 cells. When the lipid-producing cells were counted, 2 and 5% of MRG-231-6 and MRG-231-10 cells, respectively, produced lipid droplets, but virtually no lipid-producing cells were observed in MDA-MB-231 and neo-231-1 cells. When the cells were cultured in the Matrigel-coated plates, a significant increase in lipid accumulation was observed in both MRG-231 cells and MRG-negative control cells. Representative samples of lipid staining in MRG-231-6 and neo-231-1 cells are shown in Fig. 4. Fifteen percent of MRG-231-6 and 21% of MRG-231-10 cells produced lipid droplets, but only 4% of MDA-MB-231 cells and 3% of neo-231-1 contained lipid droplets, which were much smaller in size than those of MRG-positive cells (Table 1).

Induction of Differentiation of Mouse Mammary Gland by MRGp. Tissue-specific expression of milk protein in mammary epithelial cells depends on contact with stromal cells and matrix proteins. To further confirm the differentiating effect of MRG on mammary gland, we used whole-organ culture of mouse mammary glands to study whether MRGp can regulate milk protein β-casein. The

result of cytoplasmic vacuolation and the presence of secretory vesicles containing milk proteins was clearly noted in the lactating gland (Fig. 1B). We found strongly positive MRG protein staining in the alveolar mammary epithelial cells from the lactating mammary gland (Fig. 1C). The expression of MRG protein was clearly detectable in the alveolar epithelial cells in all five lactating mammary glands. In contrast, either no detectable MRG protein staining or very weak MRG protein expression was visualized in eight of the nonpregnant normal breast reduction mammoplasty specimens from nulliparous women (Fig. 1D). Expression of MRG protein was absent in all 10 cases of malignant breast carcinomas (Fig. 1E).

Expression and Purification of MRGp. Active MRGp is required to test its function on mammary epithelial cells. We expressed and purified MRGp prepared from baculovirus-infected Sf9 cells (see “Materials and Methods”). When analyzed by SDS-PAGE, the purified protein showed a single band at molecular mass of 18 kDa (Fig. 2A). The purified 18-kDa protein was confirmed as MRG by Western blot using a specific anti-MRG antibody (Fig. 2B).

Induction of Differentiation of Breast Cancer Cells. To investigate whether the high level of MRG expression in the lactating alveolar mammary epithelial is an instigator or merely a by-product of mammary gland differentiation leading to milk production, we investigated whether overexpression of the MRG gene could induce differentiation. We transfected MDA-MB-231 human breast cancer cells with full-length MRG cDNA and established several MRG-expressing clones (MRG-231 clones; Ref. 1). Fig. 3A shows the MRG protein expression in MDA-MB-231 and MRG-231-6 cells, two MRG-positive clones, but not in parental MDA-MB-231 and neo-231-1 MRG-negative cells.

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**Gland Morphology and β-casein Expression**

Second pairs of mouse whole thoracic mammary glands were cultured for 6 days with or without 50 nM MRGp in medium supplemented with bovine pituitary extract, insulin, epidermal growth factor, and hydrocortisone as described in “Materials and Methods.” Fresh medium containing MRGp was added every 2 days. Half of the gland was subjected to fixation, sectioning, and histological analysis (A and B), and the other half was subjected to RNA extraction for Northern analysis of β-casein expression (C and D). Mammary gland histological analysis: A, control (×20); B, MRGp-treated (×20). The fat droplets accumulated in MRGp-treated alveolar epithelial cells were observed (arrows). Expression of β-casein mRNA (C) was analyzed by Northern blot and normalized by visualization of ribosomal bands (D). Lane 1, mammary gland from pregnant mouse as a positive control for β-casein; Lanes 2 and 3, MRGp-treated mammary glands in organ culture; Lanes 4 and 5, control untreated glands in organ culture. Mammary glands in Lanes 2 and 4 were derived from a 10-week-old virgin mouse; mammary glands in Lanes 3 and 5 were derived from a 7-week-old virgin mouse.

**DISCUSSION**

MRG, identified and cloned by a differential cDNA sequencing approach as a novel human breast cancer growth inhibitor (1), has synergistic interaction with MRGp on mammary gland morphology and β-casein expression. Second pairs of mouse whole thoracic mammary glands were cultured for 6 days with or without 50 nM MRGp in medium supplemented with bovine pituitary extract, insulin, epidermal growth factor, and hydrocortisone as described in “Materials and Methods.” Fresh medium containing MRGp was added every 2 days. Half of the gland was subjected to fixation, sectioning, and histological analysis (A and B), and the other half was subjected to RNA extraction for Northern analysis of β-casein expression (C and D). Mammary gland histological analysis: A, control (×20); B, MRGp-treated (×20). The fat droplets accumulated in MRGp-treated alveolar epithelial cells were observed (arrows). Expression of β-casein mRNA (C) was analyzed by Northern blot and normalized by visualization of ribosomal bands (D). Lane 1, mammary gland from pregnant mouse as a positive control for β-casein; Lanes 2 and 3, MRGp-treated mammary glands in organ culture; Lanes 4 and 5, control untreated glands in organ culture. Mammary glands in Lanes 2 and 4 were derived from a 10-week-old virgin mouse; mammary glands in Lanes 3 and 5 were derived from a 7-week-old virgin mouse.

**Interaction of the ω-3 PUFA DHA and MRG on Cell Growth.** Because MRG is a fatty acid-binding protein with the highest binding affinity to the ω-3 PUFA DHA, we were interested in studying whether the growth-suppressing effect of DHA is mediated in part by MRG. We first studied the effects of DHA on MRG-negative MDA-MB-231 cells. The cells were treated with DHA at doses of 2, 4, 6, 8, and 12 μg/ml for 4 days, with fresh DHA added every 2 days. A very narrow dose-dependent growth inhibition was observed for DHA (Fig. 6A). Although no significant growth inhibition was observed for DHA at a dose of 2 μg/ml, 71 and 92% growth inhibition was observed at doses of 8 and 12 μg/ml, respectively. We therefore chose the non-inhibiting DHA dose of 2 μg/ml to test its growth-regulatory effect on MRG-positive versus MRG-negative cells. As demonstrated in Fig. 6B, when the cells were treated with 2 μg/ml DHA, 55 and 47% growth inhibition was observed in MRG-231-6 and MRG-231-10 MRG-transfected cells, respectively. However, no growth inhibition was observed in MRG-negative parental MDA-MB-231 cells and neo-231-1 cells. We also studied the effect of the ω-6 fatty acid linoleic acid on the growth of MDA-MB-231 cells. At the same conditions as for the ω-3 fatty acid DHA, no significant growth effect was observed at the similar dose range between 4 to 20 μg/ml (data not shown).

To further confirm the synergistic interaction of MRG expression and DHA on growth inhibition, we treated MRG-negative MDA-MB-436 and MDA-MB-468 cells with DHA and MRGp. MRGp treatment induced dose-dependent growth inhibition in MDA-MB-436 breast cancer cells (Fig. 7A). Although no significant growth inhibition was observed when the MRGp dose was <50 nM, 10 and 14% growth inhibition was observed when cells were treated with 50 and 80 nM MRGp, respectively. At 150 nM MRGp, growth was inhibited 58%. A submaximal MRG dose of 80 nM was used to test the interaction between MRG and DHA. Treatment of MDA-MB-436 (Fig. 7B) and MDA-MB-468 (Fig. 7C) cells with 80 nM MRGp resulted in either a slight inhibition or a slight stimulation of cell growth, respectively. When the cells were treated with MRGp together with DHA, a significant synergistic growth inhibition was observed. The growth of MDA-MB-436 cells was inhibited by 63% when the cells were treated with DHA and MRGp, compared with 18% inhibition with DHA alone. Similarly, the growth of MDA-MB-468 cells was inhibited by 80% with DHA and MRGp, compared with 22% inhibition with DHA alone.
We demonstrated that (29, 30) MRG expression was associated with an irreversible postmitotic and terminally differentiated status of cells. Therefore, it seems clear that a differentiation-associated function is a common property of this structurally related subfamily of FABPs.

It is well established that the ω-3 fatty acids DHA and EPA, found in fish oil, have a suppressive effect on tumor growth and particularly on mammary tumorigenesis. Epidemiological studies (33–37) support a role for ω-3 fatty acids as adjunct therapy in the prevention and treatment of breast cancer. This protective effect of ω-3 PUFAs can be demonstrated in animal models with carcinogen-induced mammary tumors in mouse and rat and mammary xenografts in nude mice (14–19). Various mechanisms have been proposed to explain the tumor-suppressive activity of ω-3 PUFAs; of special interest are alteration of the oxidative metabolism of arachidonic acid via the cyclooxygenase pathway (35) and changes in lipoxygenase activity (reviewed in Ref. 36). Lipid peroxidation, the oxidation of long-chain PUFAs, can produce an array of secondary products of lipid oxidation that may possess cytostatic or cytolytic capacity. It has been proposed that DHA and EPA can both directly and indirectly modulate gene expression (38). The direct effects of DHA and EPA are most probably mediated by their ability to bind to positive and/or negative regulatory transcription factors, whereas the indirect effects appear to be mediated through alterations in the generation of intracellular lipid second messengers.

At present, the mechanisms by which DHA exerts its tumor suppressing activity remain controversial and unknown. As a newly identified fatty acid-binding protein and a growth differentiation factor for mammary cells, we have demonstrated here that treatment of human breast cancer cells with DHA resulted in differential growth inhibition proportional to the MRG expression in the cells: MRG-positive cells or MRGp-treated cells were much more sensitive to DHA-induced growth inhibition than MRG-negative cells or control, untreated cells. Our data suggest that the growth-suppressing activity of DHA on breast cancer cells may be mediated in part by MRG and presumably by MRG-induced differentiation. This hypothesis is also supported by a previous report that DHA has the highest binding affinity for mouse B-FABP (MRG), suggesting that the physiological ligand for MRG is the ω-3 PUFA DHA (22).

The impact of pregnancy and lactation on breast cancer risk recently has been of great interest in terms of breast cancer prevention. As hormonally related processes, it is widely accepted that pregnancy at an early age and breastfeeding reduce the risk of breast cancer (39–42). The possibility of preventing breast cancer by manipulation of these processes with hormones or dietary factors such as ω-3 PUFAs that mimic the differentiating effect is a novel and manipulable approach to breast cancer intervention and prevention. However, little is known about the regional and developmental expression of locally acting growth factors and differentiating factors in the mammary epithelium during pregnancy and lactation. Within this context, MRG could play a role in both mammary gland differentiation and ω-3 PUFA-mediated antitumor effect. The potential application of MRG as a biomarker for mammary gland differentiation to assess the efficiency of differentiation-based breast cancer chemoprevention and to predict tumor-suppressive response to ω-3 PUFAs warrants further investigation.

expression of MRG also correlates with neuronal differentiation in many parts of the mouse central nervous system (31, 32). Furthermore, blocking antibody for MRG/B-FABP can block glial cell differentiation (31). MDGI/H-FABP protein has been detected mainly in myocardium, skeletal and smooth muscle fibers, lipid and steroid-synthesizing cells of adrenals, lactating mammary gland, and terminally differentiated epithelia of the respiratory, intestinal, and urogenital tracts (6). The results provide evidence that expression of MDGI is associated with an irreversibly postmitotic and terminally differentiated status of cells. Therefore, it seems clear that a differentiation-associated function is a common property of this structurally related subfamily of FABPs.

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