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TITLE: Characterization of Novel Breast Cancer Specific Gene, BCSG1, in Human Breast Cancer Progression (97Breast)

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We recently identified and cloned a novel breast cancer-specific gene BCSCG1 by direct differential cDNA sequencing. BCSCG1 has a great sequence homology with Alzheimer disease (AD)-related neurotic protein synuclein, and thus was also named as synuclein γ (SNCG). Overexpression of SNCG in breast cancer cells led to a significant increase in motility and invasiveness in vitro and a profound augmentation metastasis in vivo. Our data suggest that the member of neurotic protein synucleins might have important functions outside the central nervous system and play a role in breast cancer progression.
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I. BACKGROUND AND SIGNIFICANCE

Identification of quantitative changes in gene expression that occur in the malignant mammary gland, if sufficiently characterized, may yield novel molecular markers which may be useful in the understanding of breast cancer development and progression (1). Within this context, we have previously reported the isolation of differentially expressed genes in the cDNA libraries from normal breast and infiltrating breast cancer using the EST-based differential cDNA sequencing approach (2, 3). Of many putative differentially expressed genes (2, 3), a breast cancer specific gene, BCSG1, which was (a) identified as a group of EST specifically expressed in mammary gland relative to other organs and was (b) high abundance in a breast cancer cDNA library but scarcely in a normal breast cDNA library, was identified as a putative breast cancer specific gene (2).

Interestingly, BCSG1 revealed no homology to any other known growth factors or oncoproteins; rather, BCSG1 revealed extensive sequence homology to AD-related neurotic proteins synucleins that are mainly expressed in brain and localized to presynaptic terminals (4-7). The pathological hallmark of AD is amyloid deposition in neurotic plaques and blood vessels (8). Two major intrinsic constituents of amyloid are a 39-43 AA peptide named Aβ component (8) and the recently identified non-Aβ component (NAC) (4). NAC precursor was cloned from a human brain library (4) and named synuclein α (SNCA) because it shares 95% sequence homology with rat synuclein. Recently, the second synuclein named synuclein β (SNCB) was cloned from human brain and has 61% identical sequence with SNCA (6). The previously identified BCSG1, which is also highly expressed in brain (2), has 54% and 56% sequence identity with SNCA and SNCB, respectively, and has been renamed as synuclein γ (SNCG) (9). Thus, the previously unrecognized homology between these proteins defines a family of human brain synucleins that currently has three members. Although synucleins are abundant proteins expressed in presynaptic terminals and tightly associated with amyloid plaque in AD and Lewy body in Parkison's disease (PD) (10), their functions have not been defined yet. SNCA aggregation may be important in the etiology and pathogenesis in neurodegenerative disorders such as AD and PD (10). Being identified as a breast cancer specific gene, we previously demonstrated a stage-specific SNCG expression as follows: SNCG was undetectable in normal or benign breast lesions, showed partial expression in ductal carcinoma in situ, but was expressed at an extremely high level in advanced infiltrating breast cancer. In the current studies, the effects of SNCG on breast cancer growth and metastasis were investigated.

II. WORK ACCOMPLISHED

The overall hypothesis to be evaluated is that up-regulation of BCSG1/SNCG expression may indicate breast cancer malignant progression from a benign breast or a low grade in situ carcinoma and to a highly infiltrating carcinoma. The overexpression of BCSG1 may correlate with clinical aggressiveness of breast cancers. Therefore an alternations of BCSG1 expression may lead to an abnormal growth and malignant progression.

Overexpression of SNCG in breast cancer cells led to a significant increase in motility and invasiveness in vitro and a profound augmentation metastasis in vivo. This is the first report indicating the potential involvement of synuclein in the non-neurotic disease. An elucidation of the reasons for SNCG overexpression in infiltrating breast cancer and SNCG-induced metastasis may shed some light on the pathogenesis of not only breast cancer progression but also neurodegenerative disorders. Please see attached paper for detail description.
III. TRAINING
This is PI's first independent grant. The proposed studies of the current grant application includes a variety of different aims and experiments ranging from basic molecular biology, cell biology, in vivo orthotopic nude mice model for tumor growth and metastasis, and a clinical oriented study on screening clinical human breast specimens. This is the first time that PI has a chance to independently carry out a very challenging, yet ambitious, multi display project. During the last year, PI has gained a lot of experience on animal model and in vivo analysis of tumor metastasis. The success on the current grant proposal will encourage and facilitate PI's future career development as an independent clinically oriented breast cancer investigator.

IV. REFERENCES
Stimulation of Breast Cancer Invasion and Metastasis by Synuclein γ¹

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ABSTRACT

We recently identified and cloned novel breast cancer-specific gene BCSG1 by direct differential cDNA sequencing. BCSG1 has a great sequence homology with the Alzheimer's disease-related neuronal protein synuclein (SNC); thus, it was also named SNC-γ. Overexpression of SNC-γ in breast cancer cells leads to a significant increase in motility and invasiveness in vitro and a profound augmentation of metastasis in vivo. Our data suggest that this member of the neural protein SNCs might have important functions outside the central nervous system and may play a role in breast cancer progression.

INTRODUCTION

If sufficiently characterized, the identification of quantitative changes in gene expression that occur in the malignant mammary gland may yield novel molecular markers that may be useful in understanding breast cancer development and progression (1). Within this context, we have previously reported the isolation of differentially expressed genes in cDNA libraries from normal breast tissue and infiltrating breast cancer using the expressed sequence tag-based differential cDNA sequencing approach (2, 3). Of the many putative differentially expressed genes (2, 3), BCSG1, which was identified as a group of expressed sequence tags specifically expressed in the mammary gland relative to other organs and abundantly expressed in a breast cancer cDNA library but scarcely seen in a normal breast cDNA library, was identified as a putative breast cancer-specific gene (2).

Interestingly, BCSG1 revealed no homology to any other known growth factors or oncogenes; however, BCSG1 revealed extensive sequence homology to the AD²-related neuronal proteins called SNCs that are expressed mainly in the brain and localized to presynaptic terminals (4–7). The pathological hallmark of AD is amyloid deposition in neuritic plaques and blood vessels (8). Two major intrinsic constituents of amyloid are a 39–43-amino acid peptide named the Aβ component (8) and the recently identified non-Aβ component (4). The non-Aβ component of the AD precursor was cloned from a human brain library (4) and named SNCA because it shares a 95% sequence homology with rat SNC. Recently, a second SNC named SNCB was cloned from human brain, and it has a 61% sequence identity with SNCA (6). The previously identified BCSG1, which is also highly expressed in the brain (2), has a 54 and 56% sequence identity with SNCA and SNCB, respectively, and has been renamed SNCG (9). Thus, the previously unrecognized homology between these proteins defines a family of human brain SNCs that currently has three members. Although SNCs are abundant proteins expressed in presynaptic terminals and are strongly associated with amyloid plaque in AD and Lewy body in PD (10), their functions have not yet been defined. SNCA aggregation may be important in the etiology and pathogenesis of neurodegenerative disorders such as AD and PD (10). During its identification as a breast cancer-specific gene, we previously demonstrated stage-specific SNCG expression as follows: (a) SNCG was undetectable in normal or benign breast lesions; (b) SNCG showed partial expression in ductal carcinoma in situ; and (c) SNCG was expressed at an extremely high level in advanced infiltrating breast cancer. The effects of SNCG on breast cancer growth and metastasis were investigated in the current studies.

MATERIALS AND METHODS

Transfection. Full-length SNCG cDNA was inserted into a pCI-neo mamman expression vector, and the resulting vector was transfected into MDA-MB-453 cells as described previously (3, 11).

Preparation of CM. All of the clones were maintained in subconfluent monolayers with 10% FCS. The medium was discarded, and the monolayers were washed twice with PBS. The monolayers were cultured in the absence of serum in DMEM supplemented with transferrin (1 mg/liter), fibronectin (1 mg/liter), and trace elements (Biofluids, Rockville, MD). After 24 h, the serum-free medium was discarded, and the cells were replenished with the fresh serum-free medium. The CM were collected 30 h later. Media were then centrifuged at 1,200 × g, and the supernatants were saved and concentrated approximately 5-fold using an Amicon hollow fiber concentrator with a M, 10,000 cutoff at 4°C. The protein concentrations of CM were determined and normalized.

MMP Activity. The MMP enzymatic activity of the CM was assayed using a quenched fluorescent substrate Mea-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (Bachem) as described previously (12). The CM were pretreated with APMA for activation (13).

In Vitro Invasion and Motility Assay. As described previously (11), cell invasion and motility were analyzed in a modified Boyden chamber assay using 8-μm polycarbonate membranes coated with 4 mg/ml growth factor-reduced Matrigel.

Tumor Growth in Athymic Nude Mice. A tumorigenic assay was performed in nude mice as described previously (3, 11). Briefly, approximately 0.4 × 10⁶ cells (0.15 ml) were injected into a 5–6-week-old female athymic nude mouse (Friederich 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. Tumor size was determined at weekly intervals by three-dimensional measurements (in millimeters) using a calliper. Only measurable tumors were used to calculate the mean tumor volume for each tumor cell clone at each time point. Animals were sacrificed 32–40 days after injection, when the largest tumors reached about 15 mm in diameter.

Assessment of Regional Lymph Node and Lung Metastasis. As described previously (11), the axillary lymph nodes and lungs of sacrificed animals were excised, weighed, fixed in formalin, embedded in paraffin, and stained with H&E for a microscopic examination for morphological evidence of tumor metastasis. Sections were reviewed and scored by two pathologists.

Antibody Production. The purified synthetic SNCG peptide corresponding to amino acids 101–117 (2) was conjugated and injected into New Zealand rabbits as reported previously (12). The antisera was purified using SNCG peptide affinity chromatography.
RESULTS AND DISCUSSION

Transfection of SNCG into MDA-MB-435 Human Breast Cancer Cells. To determine the effects of SNCG on invasion/metastasis, we selected MDA-MB-435 human breast cancer cells as recipients for SNCG-mediated gene transfection due to their lack of detectable SNCG transcript (2) and their highly tumorigenic and aggressive phenotype in nude mice (11). Cells were transfected with a plasmid vector containing a neomycin resistance gene (neo) clones or with the same vector containing full-length SNCG cDNA (SNCG clones). MDA-MB-435 clones expressing SNCG were designated as SNCG-435 clones, and the control neo-transfected cells were designated as neo-435 clones. Fig. 1 shows the Northern blot and Western blot analyses of SNCG expression in selected clones. All selected SNCG-435 clones expressed SNCG mRNA transcripts and proteins. In contrast, none of the neo-435 clones produced any detectable SNCG transcripts and proteins. No changes in morphology were observed in these clones. Based on the level of SNCG expression, we selected SNCG-435-1, SNCG-435-3, neo-435-1, and neo-435-2 clones for the subsequent studies.

In Vitro Growth of SNCG-transfected Cells. To determine whether SNCG overexpression affects the growth of MDA-MB-435 cells, cells from exponentially growing cultures of different MDA-MB-435 clones were seeded in triplicate at 3000 cells/well (24-well plate) in 1 ml of DMEM-5% serum. The growth rates of SNCG-positive SNCG-435-1 and SNCG-435-3 cells were compared with those of SNCG-negative neo-435-1 and neo-435-2 cells in a monolayer culture. No significant differences in growth rate were observed among SNCG-positive and SNCG-negative cells (data not shown).

Metastasis in the Orthotopic Nude Mouse Model. Because SNCG was highly expressed in the infiltrating breast cancer cells relative to benign or noninvasive in situ carcinomas (2), we were interested in studying whether SNCG is an instigator of metastasis or merely a correlative product during breast cancer progression. The effect of SNCG expression on metastasis was assayed in an in vivo orthotopic (mammary fat pad) nude mouse model. Two independent experiments were done to confirm reproducibility, and the data from these experiments are summarized in Table 1. After a lag phase of 10 days, mice given implants of both SNCG-positive and SNCG-negative cells developed tumors. There was no difference in tumor incidence between neo-435 and SNCG-435 clones. Starting at about 20 days after inoculation, tumor necrosis was observed in tumors derived from SNCG-435-1 and SNCG-435-3 cells. Neo-435-1 and neo-435-2 cells also developed some tumor necrosis, but with less intensity. Consistent with the similar in vitro growth rates, there was no significant difference in primary tumor size between the neo-435 and SNCG-435 clones at 40 days after injection.

To study tumor dissemination, axillary lymph nodes and lungs were examined physically at autopsy and then subjected to microscopic examination for morphological evidence of tumor cells by light microscopy on H&E-stained paraffin sections. For the axillary lymph node, the average weight was 15 mg for neo-435 mice and 44 mg for SNCG-435 mice. The increased lymph node weight reflects the invaded breast tumors. Representative H&E-stained sections for neo-435 and SNCG-435 lymph nodes are shown in Fig. 2, A and B. Microscopic examination indicated that SNCG-435-1 and SNCG-435-3 mice showed a significantly higher average lymph node positivity (64 and 77%) compared to that (27%) of SNCG-negative neo-435-1 and neo-435-2 mice (Table 1). For lung metastases, the numbers of visible nodules on the surface of the lungs increased dramatically from an average of 1 for neo-435 mice to an average of 23 for SNCG-435 mice (Table 1). The representative lungs were shown in Fig. 2C. When these lungs were examined microscopically, large numbers of micrometastases were observed in SNCG-435 mice; the lungs of neo-435 mice had significantly fewer micrometastases (data not shown). Representative H&E-stained sections for neo-435 and SNCG-435 lungs are shown in Fig. 2, D-G. To our knowledge, human breast cancer cells usually do not form such a profound regional and metastatic tumor dissemination (visible lung nodules) in the spontaneous mammary fat pad nude mouse model. This dramatic SNCG-stimulated metastasis suggests a role for SNCG as a key positive regulator for breast cancer invasion and metastasis. The overexpression of SNCG in malignant infiltrating breast epithelial cells compared to the low expression level in noninvasive in situ carcinoma (2) suggests that SNCG expression is a meaningful marker for breast cancer malignant progression and may signal the more
Fig. 2. Axillary lymph nodes and lung metastasis from neo-435 mice and SNCG-435 mice. The mice were sacrificed at day 40 after cell injection. Lymph nodes and lungs were isolated, and some were subjected to H&E staining. Representative axillary lymph nodes from a neo-435-1 mouse (A) and a SNCG-435-3 mouse (B) are shown. Arrow, an invasive breast tumor that mainly occupied the lymph node in a SNCG-435-3 mouse. A and B, ×10. C, representative lung metastases from mice injected with SNCG-positive and SNCG-negative cells. The left lung was from a neo-435-1 mouse, and the right lung was from a SNCG-435-3 mouse. The metastatic tumors only reflect the nodules on the surface of the lungs (×2.5). D–G, microscopic examination of representative lung metastases in H&E-stained sections. D, a lung without metastases from a neo-435-1 mouse. E, a lung with micrometastases from a neo-435-2 mouse. F, a lung with a small breast tumor nodule from a SNCG-435-1 mouse. G, a lung with a large breast tumor nodule from a SNCG-435-3 mouse. Arrows, breast tumors or cancer cells. D–G, ×20.
advanced invasive/metastatic phenotype of human breast cancer. In this regard, the up-regulation of SNCG expression may facilitate breast cancer progression leading to metastasis.

MMP Activity. In an effort to investigate the molecular mechanisms underlying SNCG-induced metastasis, we studied several invasion-related factors, including MMP and cell motility. The amyloid protein has recently been demonstrated to be a strong stimulator of MMP-2 and MMP-9 expression in astrocytes (14). It is well established that the overproduction and unrestrained activity of MMPs, particularly MMP-2 and MMP-9, are linked to the malignant conversion of a variety of different tumor cells (15–22) including mammary tumors (18–22). It is interesting to test whether SNCG, an amyloid-related protein, stimulates MMP-2 and MMP-9 expression in breast cancer cells and leads to the more metastatic phenotype. We investigated whether SNCG overexpression would increase MMP activity in MDA-MB-435 cells. In this regard, the pooled CM from two SNCG-negative cells and the pooled CM from two SNCG-positive cells were concentrated and subjected to a MMP enzymatic assay. As shown in Fig. 3, no significant differences in the basal levels of proteolytic activities were observed between neo-435 and SNCG-435 clones. Mammalian MMPs are usually secreted as latent proenzymes (zymogens) and require activation for their enzymatic activity. The incubation of CM with the MMP activator organonemairial compound APMA resulted in an approximately 2-fold increase in proteolytic activity for the CM from both neo-435 and SNCG-435 clones. However, no significant difference in APMA-activated MMP activities was observed between neo-435 and SNCG-435 clones. Because the measured enzymatic activity represents the net MMP activity, reflecting the balance between activated MMPs and the tissue inhibitors of metalloproteinase, our data suggest that SNCG-induced metastasis may not be mediated by the regulation of MMP and tissue inhibitors of metalloproteinase.

**Stimulation of Invasiveness and Motility of MDA-MB-435 Cells by SNCG.** We used an *in vitro* reconstituted basement membrane (Matrigel) invasion assay to determine the effect of SNCG on cell invasion. All three SNCG-negative cells (parental MDA-MB-435, neo-435-1, and neo-435-2) were moderately invasive. At the end of a 48-h incubation, an average of approximately 250 SNCG-negative cells had crossed the Matrigel barrier. A significant stimulation of invasiveness was noted in two SNCG-positive clones, with a 3-fold increase for SNCG-435-1 cells and a 4.3-fold increase for SNCG-435-3 cells (Fig. 4A). We also investigated the effect of SNCG on cell migration without Matrigel. A similar SNCG-stimulated pattern of migration was observed. At the end of a 24-h incubation, SNCG-435-1 cells migrated 4-fold, and SNCG-435-3 cells migrated 4.2-fold over that of average SNCG-negative cells (Fig. 4B). The similar magnitude of the invasion-stimulating and migration-stimulating activity of SNCG suggests that the increased invasion in SNCG clones may be mediated by an alteration of cell motility. To determine whether the increased cell motility is mediated by chemotaxis due to the different concentrations of serum or chemoattractants in the top and bottom chambers, we compared the migration of SNCG-435-3 and neo-435-1 cells under three different culture conditions: (a) serum-free conditions; (b) serum with gradient; and (c) serum without gradient. As shown in Fig. 5, although the migration was relatively...
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low under serum-free conditions, there was a 2.8-fold increase in migration in SNCG-435-3 cells compared with that in neo-435-1 cells. When 2% serum was added in the top chamber, the migration of both SNCG-positive and -negative cells increased significantly. However, the migration of SNCG-435-3 cells was not affected by the serum gradient. Approximately 1600 of the SNCG-435-3 cells that migrated into the bottom chamber contained either 2% serum or 10% serum. These data suggest that the increased migration in SNCG-positive cells is not likely to be mediated by chemotaxis but rather by high motility features intrinsic to the cells.

Many breast tumors go through a series of events from the time of initial detection to the formation of the lethal invasive and metastatic stage. According to the three-step hypothesis of invasion (23), cell adhesion, local proteolysis, and subsequent migration or motility are key steps in the traversal of the basement membrane and connective tissue. In this study, we provide evidence linking the overexpression of neural protein SNCG, a previously identified breast cancer-specific gene (2), in human breast cancer cells with increased motility and invasive activity in vitro and a profound augmentation of metastasis in vivo.

SNCG proteins have a structural resemblance to apolipoproteins but are abundant in the neuronal cytosol and are present in enriched amounts at presynaptic terminals (9). SNCs have been specifically implicated in two diseases: AD and PD. In AD patients, a peptide derived from SNCA forms an intrinsic component of plaque amyloid (9). In PD patients, a SNCA allele is genetically linked to several independent familial cases, and the protein appears to accumulate in Lewy bodies (9). The general significance of the involvement of neural protein SNCG in cancer metastasis is unknown. Recently, SNCA and SNCB were identified as two abundant proteins through their reactivity with a monoclonal antibody recognizing MAP-2 (6) on immunoblots. In eukaryotic cells, microtubules, actin, and intermediates filaments interact to form the cytoskeletal network involved in the determination of cell architecture, mitosis, differentiation, and motility (24). Cytoskeletal organization and dynamics depend on protein self-associations and interactions with regulatory elements such as MAPs. There is increasing evidence that MAPs, including MAP-2, play a critical role in inducing microtubule assembly and controlling the dynamic instability of microtubules, thus controlling the state of their organization and assembly in cells (reviewed in Ref. 24). SNCG may interact with MAPs and regulate the cytoskeletal organization and dynamics, leading to increased motility. Nevertheless, our data indicate that the increased expression of SNCG correlates with breast cancer progression (2) and leads to a more malignant metastatic phenotype. We also demonstrated that SNCG expression in breast cancer cells was subjected to cytokine regulation and dramatically suppressed by the tumor growth inhibitor OM, and that this OM-induced transcriptional suppression of the SNCG gene was associated with OM-mediated growth inhibition. OM is an antitumor cytokine produced mainly by activated T cells and macrophages (25), and its growth-suppressing activity has been well studied in breast cancer cells (26-28). One characteristic of the host response to tumor progression is the infiltration of tumors by macrophages and T lymphocytes. The production of tumor-suppressing cytokines in a timely and locally (in situ) released fashion may represent an important function of the host defense system in suppressing tumor progression. From this prospective view, the dramatic suppression of SNCG expression in malignant breast cells by OM may represent the host-mediated tumor suppression leading to the inhibition of breast cancer progression.

This is the first report indicating the potential involvement of SNCG in a non-neural disease. An elucidation of the reasons for SNCG overexpression in infiltrating breast cancer and SNCG-induced metastasis may shed some light on the pathogenesis of breast cancer progression as well as neurodegenerative disorders.

REFERENCES


