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ORIGINAL ARTICLE

The alpha9 Nicotinic Acetylcholine Receptor is the Key Mediator in Nicotine-enhanced Cancer Metastasis in Breast Cancer Cells

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Background/purpose: The tobacco-specific mitogen nicotine was reported to correlate with cancer progression and tumorigenesis in breast cancer. Metastasis is a major cause of cancer death, so the influence of nicotine on breast cancer cell migration is also of interest. Our aim is to elucidate the mechanisms of nicotine-enhanced migration of breast cancer cells and thereby achieve better control of metastasis.
Methods: The influence of nicotine on breast cancer cell migration was evaluated by trans-well and wound-healing migration assays. Receptor-mediated migration was studied with both a small molecule inhibitor and small interfering RNA (siRNA).
Results: The alpha9 nicotinic acetylcholine receptor, α9nAChR, was identified in breast cancer cell lines MCF-7 and MDA-MB-231. Nicotine enhanced cell migration in both trans-well and wound-healing migration assays. We used a specific inhibitor and siRNA to demonstrate that α9nAChR is the key modulator in mediating nicotine-enhanced breast cancer cell migration through up-regulation of fibronectin and vimentin.
Conclusion: Nicotine treatment enhanced breast cancer metastasis through α9nAChR signaling via enhanced fibronectin and vimentin expression.

1. Introduction

Breast cancer is an important health issue worldwide. It is the most common malignancy in women.[1] Approximately 207,090 women were diagnosed with breast cancer in 2010 in the United States alone, representing approximately 28% of all female malignancies.[2] This number is 2-fold higher than the second-ranked malignancy in women, lung cancer (14%).[2] In Taiwan, breast cancer is characterized by increasing incidence and the fact that patients are of relatively young or median age (45–49 years) at diagnosis.[3] Most of the patients were diagnosed with early-stage breast cancer, which can be treated with combinations of surgery, chemotherapy and radiotherapy. However, approximately 30% of early breast cancer patients progress to develop metastatic breast cancer in the following years. With late-stage metastatic breast cancer, patients have a limited choice of further therapies and greatly diminished chances of cure.[4–6] Thus, the identification of factors that predispose to cancer metastasis will be helpful in reducing metastasis and improving the survival rate.

Tobacco smoke contains over 4000 chemical compounds, many of which are carcinogenic or mutagenic.[7–9] Nicotine is major component that is present in tobacco. It functions on the central nervous system and is responsible for addiction to tobacco.[10,11] Many studies have shown that nicotine may also enhance the progression of several different cancers, including lung, colon, gastric and breast cancers.[12–21] In general, nicotine interacts with nicotinic acetylcholine receptors (nAChRs) to activate downstream signaling pathways that promote cancer progression and metastasis.[22–25]

Structurally, nAchRs are pentamers composed of combinations of 17 subunits (α1–10, β1–β4, ε and δ).[26] The majority of nAchRs are heteropentamers, and only subunits α7 to α10 can form

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homopentamers.[27] The nAchRs are most frequently found in neuronal synapses and they mediate fast synaptic transmission. In non-neuronal tissues, the expression of nAchRs has also been demonstrated by immunoblotting, real-time polymerase chain reaction (PCR) and flow cytometry, particularly in the bronchial epithelium, endothelium and skin keratinocytes. However, the actual functions of the nAchRs in non-neuronal tissues remain unclear.[8,28]

nAchRs have been noted to promote a nicotine-stimulated epithelial-to-mesenchymal transition (EMT) in a variety of human cancer cell lines, especially in small cell lung cancer and non-small cell lung cancer.[12,13] Several studies have reported roles of nAchRs in carcinogenesis, proliferation, angiogenesis, inhibition of apoptosis and metastasis in breast, lung and colon cancers.[14–17,29,30] In studies of colon cancer and gastric cancer, α7-nAChR was found to play a dominant role in mediating cancer progression and metastasis.[29,30] Recently, some reports have shown that α9nAChR may be the key molecule in mediating nicotine-induced tumorigenesis and progression.[17,31,32] Several studies have also revealed that nicotine can enhance the metastatic behaviors of breast cancer cells.[15,22] It is not clear, however, which type of nAChRs are the key molecules in mediating nicotine-enhanced breast cancer metastasis.

This study aimed to demonstrate that nicotine exposure enhances breast cancer cell migration and that pre-treatment with a nAChR inhibitor could block nicotine-enhanced breast cancer migration. We also aimed to find out whether α9nAChR plays a key role in nicotine-enhanced breast cancer migration.

2. Materials and methods

2.1. Reagents and drugs

Nicotine, methyllycaconitine, crystal violet, G418 and Trizol reagent were purchased from Sigma-Aldrich (St Louis, MO). Anti-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-α9nAChR antibody was purchased from Abcam (Cambridge, MA).

2.2. Cell culture

Two human breast cancer cell lines, MCF-7 and MDA-MB-231, were purchased from the American Type Culture Collection (Manassas, VA). These cell lines were cultured in DMEM/F12 (Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (Life Technologies, Grand Island, NY), 100 U/ml penicillin G and 100 μg/ml streptomycin. The cells were kept at 37 °C, 95% humidity and in 5% carbon dioxide. They were treated with nicotine, as previously described.[17] The scrambled control MDA-MB-231 and α9nAchR siRNA knockdown MDA-MB-231 cells were kindly provided by Professor Ho and generated as previously described.[17]

2.3. Real-time polymerase chain reaction

Total RNA was extracted using the Trizol reagent according to the manufacturer's instructions (Life Technologies, Grand Island, NY) and as previously described.[29,33] Two micrograms of total RNA were transcribed to cDNA in a 20 μL reaction using Moloney murine leukemia virus reverse transcriptase at 37 °C for 90 minutes. For validation, real-time-PCR was performed using the Power SYBR-Green real-time RT-PCR system and the ABI 7500 Fast™ detection system (Applied Biosystems, Foster City, CA). The primer sequences for the target genes were as follows:

- α9nAchR (forward: 5′ - AAA GATGAACTGGTCCCATTCCT -3′; reverse: 5′-AAGGTCATTAAA CAACTTCTGAGC-3′);
- E-cadherin (forward: 5′ - CGGGAATGCAGTTGAGGATC-3′; reverse: 5′ - AGGATGGTGTAAGCGATGGC-3′);
- N-cadherin (forward:5'-AACCCTTATTTTGCCCCCAAT-3′; reverse: 5'-TCAACATGGTACCGGCATGA-3′);
- fibronectin (forward: 5'-GTGTGACCCTCATGAGGCAAC-3′; reverse: 5'-CTGGCCTCCAAAGCATGTG-3′);
- vimentin (forward: 5'-GGCTCAGAT TCAGGAACAGC-3′; reverse: 5'-CTGAATCTCATCCTGCAGGC-3′); and
- β-actin (forward: 5′-AGCGCGGCTACAGCTTCA -3′; reverse: 5′-GGCCATCTCTTGCTCGAAGT-3′).

The reaction conditions were 95 °C for 3 minutes, 95 °C for 15 seconds and 60 °C for 1 minute (40 cycles). For validation, real-time-PCR was performed three times independently.

2.4. Protein extraction and western blot analysis

The cells were washed with cold phosphate buffer saline (PBS) and harvested using a cell lysis buffer (Thermo Fisher Scientific, Rockford, IL) containing protease inhibitors (Complete protease inhibitor tablets, Boehringer Mannheim, Indianapolis, IN). Equal amounts of protein from control and treated cell lysates were separated using a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel under reducing conditions and transferred onto polyvinylidene fluoride membranes (GE Healthcare, Piscataway, NJ) that were subsequently probed with anti-α9nAChR and anti-β-actin primary antibodies. Horseradish peroxidase-conjugated secondary antibodies (1:5000) were used with enhanced chemoluminescence reagent (GE Healthcare, Piscataway, NJ) to visualize the protein bands. Images of the films were captured using the VersaDoc 5000 (Bio-Rad Laboratories, Hercules, CA).[33–36]

2.5. Silencing the expression of α9nAchR in breast cancer cells

The α9nAchR-siRNA knockdown (α9nAchR KD) MDA-MB-231 cells and scrambled control MDA-MB-231 cells were from Professor Ho and were generated as previously described.[17] We diminished α9nAchR expression in MCF-7 cells using MISSION short hairpin (sh) RNA clones purchased from Sigma Chemical Co. (St. Louis, MO). MISSION shRNA clones are sequence-verified shRNA lentiviral plasmids for gene silencing in mammalian cells. The parental vector (pLKO.1<-puro) allows for transient transfection or stable selection via puromycin resistance. The target sequence for the human α9nAchR messenger RNA (NM_017581) gene was 5′-GAATGGAA-GAAGGTGGCGAAA-3′. The MISSION non-target shRNA control vector (SHC002) was used as a scrambled control and the sequence of scrambled shRNA was 5′-CAACAAGATGAAGAGCACCAA-3′. The transfection protocol has been described previously.[33,34,37–39] Briefly, 1.5×10^5 cells were washed twice with PBS and mixed with 0.5 μg of plasmids. We applied a 20 ms pulse at a fixed voltage of 1.4 kV on a neon pipette-type microporator (Life Technologies, Grand Island, NY). The stably transfected cells were selected by the appropriate antibiotic.

2.6. Migration assay

To further dissect the regulation of nicotine-enhanced cell migration, methyllycaconitine, an antagonist of nAChR, was utilized in migration assays. *In vitro* cell migration was performed in the BD Falcon cell culture insert (BD Biosciences).[29,40,41] The 1×10^5 MCF-7 or MDA-MB-231 cells that were suspended in 500 μL serum-free DMEM/F12 were seeded into the upper part of each chamber in the presence or absence of nicotine and with or without inhibitors, whereas the lower compartments were filled with 1 mL of culture