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Research paper

Neurokinin-1 receptor mediated breast cancer cell migration by increased expression of MMP-2 and MMP-14

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ABSTRACT

Breast cancer (BC) is a common reason of cancer-associated death in female. To develop novel strategy of therapeutics, it is crucial to comprehensively understand the receptor status of BC cells on the surface and inner, because chemical messengers can bind the receptors and promote tumorigenesis. Compared with normal and benign samples, BC cell lines and malignant biopsies showed higher expression of neurokinin-1 receptor (NK1). In current work, we examined the role and mechanism of NK1 receptor signaling in BC cell migration. Human hemokinin-1 (hHK-1) was the peripheral agonist of NK1 receptor. Our results showed that by activating NK1 receptor, hHK-1 promoted the migration of BC cells. Gelatin zymography and WB experiment showed that hHK-1 enhanced the levels of MMP-2 and MMP-14; inhibition of these two MMPs blocked hHK-1-induced cell migration. We further explored the underlying mechanism. hHK-1 incuced the phosphorylation of ERK1/2, JNK and Akt through PKC or PKA pathway. The phosphorylation of these kinases further regulated the activation of transcriptional factor AP-1 and NF-κB. Inhibition of AP-1 and NF-κB reduced the up-regulation of MMP-2 and MMP-14 by hHK-1. Taken together, we showed NK1 receptor was an important regulator of human BC cell migration and a potential target for BC treatment.

1. Introduction

Breast cancer (BC) remains the general reason of cancer-associated mortality among women. Most patients with BCs did not die due to the primary tumor but the distant metastasis of the carcinoma (Smid et al., 2008; Walker et al., 2013). In order to improve current therapeutics, it is necessary to explore the complex mechanisms of BC metastasis and identify new pharmalogical target.

Mammalian tachykinins refer to a group of small bio-active peptides sharing the common C-terminal region of –FXGLM-NH_2 where X is hydrophobic amino acid. This family included three members, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), until 2000, hemokinin-1 was identified as the fourth mammalian tachykinin (Pennefather et al., 2004). Both SP and HK-1 is selective agonist of NK1 receptor (Bellucci et al., 2002). However, unlike that SP is a classical neurotransmitter of neuronal origin, HK-1 is primarily expressed in hematopoietic and immune cells thus being considered as a peripheral agonist of NK1 receptor (Zhang et al., 2000; Satake and Kawada, 2006).

Traditionally NK1 receptor and its agonist are related with the physiological processes of emotion, vomit reflex, pain transmission, vasodilation and inflammation (Pennefather et al., 2004; Satake and Kawada, 2006). In recent years, a relationship between NK1 system and tumor development is established. The aberrant expression of NK1 receptor has been identified in various tumor tissues such as glioblastoma, breast, pancreatic and melanoma (Palma, 2006; Munoz et al., 2011; Reddy et al., 2008). In these tumor tissues, SP and HK-1 can be produced by tumor cells themselves or by immune cells, endothelial cells and neuronal cells which are present surrounding tumor cells (Satake and Kawada, 2006; Palma, 2006). Reciprocally, these peptides can contribute to the alteration of tumor microenvironment by modulate leukocyte trafficking, cytokines release and angiogenesis (Feistritzer et al., 2003; Schratzberger et al., 1997; Ruff et al., 1985; Song et al., 2012). They can also directly activate the NK1 receptor on tumor cells, mediating tumor cell proliferation and anti-apoptosis effect (Recio et al.,

Abbreviations: AP-1, activator protein-1; NF-κB, nuclear factor kappa B; ERK1/2, extracellular regulated protein kinases1/2; JNK, c-jun N-terminal kinase.

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2013; Mitsuhashi et al., 1992; Lieb et al., 1997; Christian et al., 1994; Luo et al., 1996; Akazawa et al., 2009).

The relationship of NK1 system and breast cancer has been widely reported. In breast cancer (BC), compared with normal or benign breast biopsies, a number of BC cell lines and malignant breast biopsies were shown to over-express NK1 receptor and the expression level seemed to be related with the malignant degree of tumors (Castro et al., 2005; Rao et al., 2004; Singh et al., 2000). The agonist of NK1 receptor promoted BC cells growth and the NK1-selective antagonists blocked the effects (Bigioni et al., 2005; Huang et al., 2010). Garcia-Recio et al. reported that SP trans-activated HER2 and affected cellular responses to anti-ERBB therapies in BCs (Recio et al., 2013). SP was shown to directly regulate MDA-MB-468 cell migration possibly by an increased expression of α2 integrin (Lang et al., 2004). Castrol et al. reported that the expression of NK1 and preprotachykinin-1 in breast cancer cells depended on the relative degree of invasive and metastatic potential (Castro et al., 2005). In an *in vitro* coculture model, NK1 system facilitated the bone marrow metastasis of BC cells (Rao et al., 2004). These evidences pointed out a possible important role of NK1 receptor in BC migration and invasiveness, but little is known about the detailed molecular mechanism of this process.

NK1-mediated cell migration has been described in different tumor types such as glioma and pancreatic cancer cells involving the regulation of matrix metalloproteinase (MMP) activity (Mou et al., 2013; Li et al., 2013). In BC cells, MMPs have been closely associated with the invasive process of BC cells (Egeblad and Werb, 2002; Chabottaux and Noel, 2007; Duffy et al., 2000). High MMP-2 level is found to be a breast cancer metastasis indicator and correlate with poor outcome in BC patients (Pellikainen et al., 2004; Jezierska and Motyl, 2009). MMP-14 was a membrane type MMP (also named as MT1-MMP) which processed newly-synthesized pro-MMP-2 into active MMP-2 (Nishida et al., 2008). Both MMP-2 and MMP-14 degraded the ECM, leading to invasion and metastasis of BC cells (Chenard et al., 1999; Azzam et al., 1993).

Based on these findings, this study aimed to investigate the signaling pathways of NK1 receptor in BC cells and how they would be involved in BC cell migration. We showed that NK1 receptor mediated BC cell migration by enhancing the level of MMP-2 and MMP-14. This effect was involved with signaling pathway composed of activated ERK1/2, JNK, Akt, NF-κB and AP-1. These results indicated a correlation of NK1 system with the MMPs expression as well as the migratory ability of BC cells, suggesting that NK1 system may be a potential therapeutic target to intervene BC metastasis.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS), DMEM medium, RPMI 1640 medium, Opti-MEM medium, transfection reagent Lipofectamine2000 and cell dissociation buffer were from Invitrogen (Life Technologies, Carlsbad, CA, USA). PD98059 (MEK inhibitor), LY294002 (IP3K inhibitor), Cell-Titre blue cell viability assay and luciferase assay system were from Promega (Madison, WI, USA). The enhanced chemoluminescence (ECL) detection system and BCA protein assay kit were from Pierce (Thermo, Rockford, IL, USA). SP600125 (JNK inhibitor), gelatin, the selective antagonists L-732138 (NK1), SR48968 (NK2) and SB22200 (NK3) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). AP-1 inhibitor Curcumin and tanshinone IIA, NF-κB inhibitor caffeic acid phenethyl ester (CAPE) and pyrrolidinedithiocarbamic acid (PDTC) were from Sangon (Shanghai, China). PVDF membrane, protease inhibitor cocktail and phosphatase inhibitor cocktails were from Roche (Pleasanton, CA, USA). 5 × Laemmli buffer, non-denaturing 5 × Laemmli buffer, RIPA lysis buffer, luciferase reporter gene p-NFκB-Luc and p-AP1-Luc were from Beyotime (Jiangsu, China). Antibodies against phospho-ERK1/2, ERK1/2, phospho-SAPK/JNK, SAPK/JNK, pAkt, Akt, phospho-p65, p65, phospho-c-JUN, c-JUN, GAPDH and HRP-conjugated secondary antibody were from Cell Signaling Technology Inc. (Danver, MA, USA). The antibodies against MMP-2, MMP-14 and human NK1 were from Abcam (Cambridge, UK).

2.2. Cell culture and peptides synthesis

Human breast cancer cell line MCF-7, MDA-MB-231 and T47D was purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI1640 supplemented with 10% FBS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) in a humidified atmosphere of CO_2/air (5%/95%) at 37 °C.

Human hemokinin-1 (hHK-1) peptide (TGKASQFFGLM-NH_2) and FITC-conjugated hHK-1(FITC-hHK-1) were synthesized by Fmoc solid-phase method as described before (Friedl, 2011). The characteristics of the peptide were confirmed by ESI-TOF mass spectrometry. The purity of peptide was quantified to be >95% using reversed-phase HPLC by a C18 column as the solid phase and a H_2O: acetonitrile gradient as the liquid phase.

2.3. Transwell assay

Cell migration assays were performed with the transwell inserts (8 μm pore size; Corning, NY, USA). Briefly, cells were digested with cell dissociation buffer. 8×10^4 cells in 0.1 ml of serum-free RPMI1640 medium were seeded in the upper chamber, and 0.6 ml of the same medium with or without hHK-1 was added in the lower chamber. After incubation at 37 °C for 24 h, cells were fixed with 90% EtOH for 30 min and stained with 0.1% crystal violet in PBS for 15 min. The non-migrant cells were rubbed off from the upper face of the transwell membrane with a cotton swab. The stained cells were solved in 10% acetic acid for 15 min. The absorbance values were determined at 600 nm on a FlexStation III plate-reader (Molecular Devices Corp., Palo Alto, CA, USA). To measure the effect of inhibitors, cells were pre-treated for 30 min with different inhibitors at the concentrations indicated. The migration fold of the cells in each experiment was corrected by the cell viability assay.

2.4. Cell viability measurement

Cell viability was determined by Cell-Titre blue assay. Cells were seeded in 96-well plate at a density of 10,000/well. The cells were treated with different chemicals for 24 h. After the incubation, Cell-Titre blue reagent was added for 4 h at 37 °C. The fluorescence was measured on FlexStation III plate reader (emission: 590 nm; excitation: 560 nm).

2.5. Western-blotting analysis

Cells were seeded in 12-well plate at a density of 250,000/well. After the treatment, cells were rinsed with PBS and then lysed by RIPA buffer with protease and phosphatase inhibitor cocktails. The lysates were centrifuged at 15,000*g* for 10 min at 4 °C. To measure the effect of inhibitors, cells were pre-treated for 30 min with different inhibitors at the concentrations as indicated in figures. The supernatant was collected and the protein concentration was determined with BCA reagent. 30 μg of protein from each sample was electrophoresed on 10% SDS-PAGE gel and transferred onto a PVDF membrane. The membranes were probed with primary antibodies including phospho-ERK1/2 (1:1000), phospho-SAPK/JNK