Jun Amino-terminal Kinase 1 Activation Promotes Cell Survival in ErbB2-Positive Breast Cancer

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Abstract. Background: Downstream signaling is a key component of Her2/neu overexpression in human breast cancer. Major survival pathways downstream of Her2/neu include mitogen and stress activated protein kinases (ERK, JNK, p38). Materials and Methods: MAPK protein expression was examined in mouse and human cancer tissue. MAPK expression was inhibited by genetic and pharmacologic methods in human breast cancer cell lines. The effects of MAPK inhibition on tumor formation in a preclinical model were determined. Results: It was shown that tumors from MMTV-neu mice expressed high levels of activated JNK1. Levels of this kinase were also highest in Her2/neu overexpressing human breast cancer cell lines. JNK1 inhibition specifically induced apoptosis in these lines. A JNK1 inhibitor also increased the latency period and decreased growth of MMTV-neu tumors by induction of apoptosis. JNK1 was preferentially activated in human breast cancer tissue overexpressing Her2/neu. Conclusion: JNK1 promotes cell survival in Her2/neu-positive breast cancer.

Oncogene addiction refers to the fact that a cancer cell, despite numerous genetic alterations, displays dependence on a single oncogenic pathway for proliferation or survival (for review see 1). Inactivating these pathways in normal cells is often tolerated, suggesting that oncogene addiction is a unique feature of cancer. Inhibition of these pathways in cancer cells may decrease proliferation and survival, giving rise to new therapeutic opportunities. Many human tumor cell lines retain dependence on activated oncogene pathways. Elimination of these signals induces growth arrest, senescence, differentiation, or apoptosis. Oncogene addiction suggests a cancer cell autonomous response to these signals which are independent of external factors. Oncogene addiction may be beneficial or essential to tumor cell survival.

Key Words: JNK1, growth factors, apoptosis, kinase, mammary cancer.

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Protein kinases acting as oncogenes are important targets for cancer therapy. These proteins are often activated by mutation or gene amplification. Chemotherapy agents targeting the ErbB family receptor ErbB2/HER2 have been developed. This gene is amplified in 30% of breast cancers (2, 3). Breast cancer cells in culture or grown as xenografts are inhibited by HER2-specific antibodies (4, 5) and small molecule inhibitors (6, 7). These findings led to successful clinical trials in patients with HER2-amplified metastatic breast cancer (8).

A number of hypotheses have been proposed to account for oncogene addiction, including genomic instability, suppression of lethal signaling interactions, and oncogene shock (9-11). Downstream signaling is a key component of oncogene addiction. Major survival pathways downstream of Her2/neu include mitogen and stress-activated protein kinases (ERK, JNK, p38) and phosphatidylinositol-3-kinase/Akt (12). In this study, it is shown that tumors from MMTV-neu mice expressed high levels of activated JNK1. Levels of this kinase were also highest in Her2/neu overexpressing human breast cancer cell lines. JNK1 inhibition specifically induced apoptosis in these lines. A JNK1 inhibitor also increased the latency period and decreased growth of MMTV-neu tumors by induction of apoptosis. JNK1 was preferentially activated in human breast cancer tissue overexpressing Her2/neu. It is concluded that JNK1 promotes cell survival in Her2/neu positive breast cancer.

Materials and Methods

Transgenic mouse procedures. Animal procedures were approved by the institutional animal care committee. The mammary tumor prone MMTV-neu transgenic strain in the FVB background was purchased from The Jackson Laboratory, Bar Harbor, ME, USA. Mice were genotyped by PCR analysis of tail DNA samples. Mice were given intraperitoneal doses of 10 mg/kg of the ERK inhibitor PD98059, JNK inhibitor SP600125, p38 inhibitor SB203580, or vehicle in corn oil daily for 24 weeks (20 mice/group). The mammary gland chains of female mice were examined visually and by palpation twice weekly. Tumors were measured twice weekly using calipers. Mice were euthanized when tumors reached 2 cm in their largest dimension. Tumor tissue was processed for histopathologic and gene expression analyses. Statistical analysis was determined by ANOVA.
Histopathology and immunohistochemistry. Paraffin-embedded human invasive ductal breast carcinoma specimens were obtained from pathology department archives. Mouse tumor tissue was fixed in formalin for 16 hours at room temperature. Tissue was dehydrated in an ethanol series followed by clearing in xylene and embedding in paraffin. Seven micrometer sections were cut from the blocks and placed on poly-L-lysine coated slides. Sections were deparaffinized in xylene and stained with hematoxylin and eosin for histopathologic interpretation. For immunohistochemistry, sections were rehydrated and blocked with 10% normal serum followed by incubation with anti-phosphorylated or total ERK1, JNK1, and p38 antibodies for one hour at room temperature. After washing in PBS, the sections were incubated with anti-mouse IgG secondary antibody conjugated to biotin for 10 minutes at room temperature. After additional washing in PBS, the cultures were incubated with streptavidin conjugated horseradish peroxidase enzyme for 10 minutes at room temperature. Following final washes in PBS, antigen-antibody complexes were detected by incubation with hydrogen peroxide substrate solution containing aminoethylcarbazole chromogen reagent. Immunohistochemical results were photographed using light microscopy. Statistical significance was determined by Student’s t-test.

Cell culture and siRNA transfection. The normal human mammary epithelial cell (HMEC) strain used in this study was purchased from Lonza, Rockland, ME, USA. The human breast cancer cell lines were purchased from the American Type Culture Collection, Manassas, VA, USA and cultured in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, and 40 μg/ml gentamicin in a humidified atmosphere of 5% CO2 at 37°C. Cultures plated at low density (10^4 cells/35 mm dish) were treated with 10 μM of the ERK inhibitor PD98059, 5 μM of the JNK inhibitor SP600125, or 5 μM of the p38 inhibitor SB203580 or vehicle for 24 hours. Cells were transfected with siRNA to ERK1, JNK1, or p38 according to manufacturer’s recommendations (Dharmacon), Lafayette, CO, USA.

Apoptosis analysis. Treated human breast cancer cell cultures were fixed with 70% ethanol at –20°C for 30 minutes and washed with PBS. Cultures or deparaffinized mouse mammary tumor tissue were incubated with terminal deoxynucleotidy transferase and fluorescein conjugated dUTP at 37°C for 30 minutes followed by washing in PBS. Apoptotic cells were visualized by fluorescence microscopy. The percentages of apoptotic and viable cells were determined by counting of 10 random high power fields.

Western blot. Protein was extracted in 1x Laemmli buffer from human breast cancer cell lines. 75 μg total cellular protein was separated by SDS-PAGE on 10% resolving gels under denaturing and reducing conditions. Separated proteins were electroblotted to PVDF membranes according to manufacturer’s recommendations (Roche Applied Science), Indianapolis, IN, USA. Blots were incubated with antibodies to total or phosphorylated ERK1, JNK1, p38, e-Jun, and JunB for 16 hours at 4°C. After washing in Tris-buffered saline containing 0.1% Tween 20 (TBST, pH 7.4), blots were incubated for 30 minutes at room temperature with anti-IgG secondary antibody conjugated to horseradish peroxidase. Following extensive washing in TBST, bands were visualized by the enhanced chemiluminescence method (Roche Applied Science), Indianapolis, IN, USA.

Reverse transcription-polymerase chain reaction. RNA was extracted from human breast cancer cell lines using a commercially available kit (Qiagen, Valencia, CA, USA) and reverse transcribed using SuperScript II reverse transcriptase according to manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). cDNA was amplified using specific primers (ERK1, 5’-AGACTCCCAAAG CCCTTGACC-3’ and 5’-TGCTGTCTCTTGAGAG-3’; JNK1, 5’-AGAGGA GAACCAAGAATTGGG-3’ and 5’-TAGTCATCTACAGCAGGCC-3’; p38, 5’-GCCCTTGACATGCCTCACTT-3’ and 5’-ACAGAA CAGAAAAACAGTGCTC-3’) in 20 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 63 mM KCI, 0.05% Tween 20, 1 mM EGTA, 50 μM of each dNTP, and 2.5 U Taq DNA polymerase (Roche Applied Science, Indianapolis, IN, USA). Amplification with β-actin cDNA using primers 5’-ACAGGAA GTCCCTTGCCATC-3’ and 5’-ACTGCTTCAAGTCAAGTG-3’ as the internal control was carried out by real-time PCR (iCycler; Bio-Rad, Hercules, CA, USA) using SYBR Green and cycle conditions 94°C for 25 seconds, 55°C for 1 minute, and 72°C for 1 minute.
Results

To determine if stress-activated protein kinases were activated in mouse MMTV-neu mammary tumors, this tissue was analyzed for expression of activated ERK1, JNK1, and p38 by immunohistochemistry. The histopathologic appearance of these tumors is shown in Figures 1A and B. The tumor tissue was composed of sheets and cords of monomorphic poorly differentiated epithelial cells. These cells exhibited large pale nuclei with abundant cytoplasm. Immunohistochemical analysis revealed no expression of activated ERK1 or p38 (Figures 1C and D). However, total and activated JNK1 expression was detected in MMTV-neu tumors. JNK1 protein was abundant in the cytoplasm of MMTV-neu tumor cells (Figure 1E), while activated JNK1 was primarily nuclear with cytoplasmic staining detected (Figure 1F). These results indicate that activated JNK1 is expressed in MMTV-neu mammary tumors.

To determine if activated JNK1 was also expressed in human breast cancer, Western blots were performed on normal human mammary epithelial cells (HMECs) and breast cancer cell lines. As shown in Figure 2, activated JNK1 was expressed in all human breast cancer cell lines but not in HMECs. High levels of JNK1 protein were expressed in breast cancer cell lines which overexpress Her2/neu (MDA-MB-231, MDA-MB-468, Hs-578-T, and SKBR3). JNK1 expression was up to 50 fold lower in human breast cancer cell lines without Her2/neu overexpression (MCF7, T47D) and was not detected in human normal mammary epithelial cells. Expression of downstream targets of JNK1 (c-jun and JunB) was also examined. Similar to JNK1 expression, c-jun protein was highest in human breast cancer cell lines which overexpress Her2/neu. c-jun protein was not detected in HMEC, MCF7, and T47D cells. JunB also was expressed in Her2/neu overexpressing lines Hs-578-T and SKBR3 but not in other lines. In contrast, high levels of activated ERK1 were expressed only in 3 of 4 human breast cancer lines overexpressing Her2/neu (MDA-MB-231, MDA-MB-468, and Hs-578-T). Activated ERK1 expression was low or undetectable in HMEC, MCF7, T47D, and SKBR3. Total ERK1 expression was highest in MDA-MB-231, MDA-MB468, Hs-578-T, and SKBR3. Total ERK1 expression was low or undetectable in HMEC, MCF7, and T47D cells. Expression of activated p38 in human breast cancer cell lines was also examined. Activated p38 was expressed in only 2 of 6 human breast cancer cell lines (MDA-MB-231 and SKBR3). Total p38 was detected in all breast cancer cell lines but not in HMECs. These results indicate that activated JNK1 was consistently expressed in human breast cancer cell lines that overexpress Her2/neu.

To determine if JNK1 was required for survival of human breast cancer cells overexpressing Her2/neu, these lines were treated with individual protein kinase inhibitors. As shown in Figure 3, treatment of low density cultures with the JNK1 inhibitor SP600125 reduced the number of viable cells in Hs-578-T, SKBR3, MDA-MB-231, and MDA-MB-468 cultures.
by >90%. In contrast, the ERK1 inhibitor PD98059 was not effective at inducing apoptosis in SKBR3 cells (30% reduction in viable cells). The p38 inhibitor SB203580 was not effective at inducing apoptosis in MDA-MB-231 cells (25% reduction in viable cells). These results indicate that JNK1 mediates key survival signals in human breast cancer cell lines overexpressing Her2/neu.

To confirm these results, ERK1, JNK1, or p38 expression were inhibited using siRNA transfection of Her2/neu overexpressing cell lines. As shown in Figure 4A, siRNAs were highly effective in inhibiting expression of these kinases (80-90% reduction in mRNA levels). Cell viability was assessed in these lines by TUNEL analysis. As shown in Figure 4B, JNK1 inhibition by siRNA reduced cell viability by 80-90% in all 4 cell lines. In contrast, ERK1

Figure 4. Inhibition of JNK1 expression consistently induces apoptosis in human breast cancer cell lines. A: Human breast cancer cell lines were treated with siRNA to ERK1, JNK1, and p38 as described in the Materials and Methods. Relative mRNA expression of these kinases was determined by quantitative RT-PCR. These experiments were performed three times with similar results. Error bars indicate SEM. B: Apoptosis was analyzed in human breast cancer cell lines transfected with siRNAs to JNK1, ERK1, and p38 as described in the Materials and Methods. These experiments were performed three times with similar results. Error bars indicate SEM.

Figure 5. Inhibition of JNK1 activity increases latency period of MMV-neu tumors in vivo. A: MMV-neu mice were injected weekly with the JNK1 inhibitor SP600125 (SP), the ERK1 inhibitor PD98059 (PD), or the p38 inhibitor SB203580 (SB) as described in the Materials and Methods. The number of weeks to palpable tumor formation was recorded. B: JNK inhibition decreases the growth rate of MMV-neu mammary tumors. The number of weeks from tumor detection to attain a 2 cm tumor in MMV-neu mice treated with PD98059, SP600125, or SB203580 was recorded. C: JNK inhibition induces apoptosis in MMV-neu tumors. The number of apoptotic cells in tumors from mice treated with PD98059, SP600125, or SB203580 was determined as described in the Materials and Methods. These experiments were performed three times with similar results. Error bars indicate SEM.
inhibition was less effective at inducing apoptosis (40-90% reduction in viable cells). Similarly, p38 inhibition was less effective at inducing apoptosis in human breast cancer cell lines (30-90% reduction in viable cells). These results confirm that JNK1 is a critically important survival signal for human breast cancer cells overexpressing Her2/neu.

To determine if JNK1 inhibition affected mammary tumorigenesis in vivo, MMTV-neu mice were treated with JNK1, ERK, or p38 kinase inhibitors. As shown in Figure 5A, treatment of MMTV-neu mice with the JNK1 inhibitor SP600125 resulted in a significantly longer latency period (31 weeks vs. 24 weeks for control; \( p<0.03 \)). In contrast, the latency period was not significantly increased when MMTV-neu mice were treated with the ERK inhibitor PD98059 or the p38 inhibitor SB203580. The growth rates of tumors in mice treated with these kinase inhibitors were examined. As shown in Figure 5B, SP600125 treatment increased the number of weeks required to reach the 2 cm tumor endpoint (17 weeks vs. 10 weeks for control; \( p<0.01 \)). In contrast, PD98059 or SB203580 treatment did not significantly increase the time period required to reach 2 cm tumors. SP600125 treatment significantly increased the number of apoptotic cells in MMTV-neu mammary tumors (16% vs. 2.5% in control; \( p<0.007 \)). PD98059 or SB203580 treatment did not significantly increase the numbers of apoptotic cells in mammary tumors (Figure 5C). These results indicate that JNK1 inhibition can significantly inhibit mammary tumorigenesis via induction of apoptosis.

To determine if JNK1 expression correlated with ErbB2 overexpression in human breast cancer, levels of these proteins were examined by immunohistochemistry in archival pathology specimens. Fifty-six cases of ErbB2-negative human breast tumors were examined. JNK1 expression was not observed in any of these cases (Figures 6A and B). In contrast, JNK1 overexpression was observed in 24 out of 35 ErbB2-positive human breast cancer cases (69% of tumors; \( p<0.001 \); Figures 6C and D). These results indicated that JNK1 expression correlated with ErbB2 overexpression in human breast cancer.

**Discussion**

The key finding of this study is that JNK1 mediates important cell survival signaling in Her2/neu positive mammary cancer in humans and a mouse model. A previous study determined that apoptosis mediated by the anti-estrogen tamoxifen is regulated by JNK1 (13). Vitamin E is a selective apoptotic agent in breast cancer cells whose effects are mediated by JNK1 (14). The JNK pathway also inhibits apoptosis in response to microtubule-interfering agents in breast cancer cells (15). JNK1 also regulates the response of breast cancer cells to doxorubicin (16, 17). Inhibition of JNK1 has been shown to inhibit proliferation of breast cancer cells lines (17). The apoptotic response to paclitaxel in breast cancer cell lines was mediated by JNK1 but not ERK1 or p38 (18). Decreased JNK1 expression observed in ErbB2-negative breast tumors has been associated with improved overall survival in the clinical setting (19). Combined overexpression of JNK1 and p38 was associated with poor overall survival in this study. Estrogen receptor inhibits cell death in human breast cancer cells by modifying c-jun activity (20). c-jun and JunB overexpression was previously demonstrated in human breast cancer specimens (21). c-jun overexpression has been shown to increase breast cancer cell motility, invasion, and tumor formation in nude mice (22). Expression of activated JNK1 and p38 have been positively associated with breast cancer metastasis in clinical specimens (23). These studies have demonstrated that JNK signaling is a critical mediator of breast cancer cell survival.

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