Research Article

Altered Tumor Necrosis Factor-Alpha Signaling Pathway between High and Low Metastatic Carcinoma Clones Derived from Single Tumor

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Abstract

Breast cancer cells typically metastasize to the lung, liver, bones, and brain. Metastasis of the cancer cells is the primary cause of death in breast cancer patients. The mechanisms of cancer cell dissemination and proliferation at the secondary organ are not very well understood. Our goal is to identify the differences in the molecular pathway between high and low metastatic murine mammary cancer cells by using gene array. The 4T1 and 67NR cell lines are clonal populations of cells derived from a mammary carcinoma arising from a BALB/cfC3H mouse. Metastasis of disseminated 4T1 or 67NR cancer cells in BALB/c mice was investigated by systemic infusion of cancer cells by tail vein or after surgical removal of a primary tumor. To gain insight into the specific molecular pathways between these two cell lines, differences in the expression of mRNA was measured using an oligonucleotide array. Three different methods: histology, cell colony and survival assays have confirmed that 4T1 cells are highly metastatic in BALB/c mice compared to 67NR cells. The 4T1 cells developed metastases in the lungs, liver, heart, kidney, spleen, bone and brain while 67NR cells only metastasized to the lung. A gene array study confirmed that TNF-α dependent and independent apoptotic pathways are down regulated in 4T1 cells. Furthermore, a dose dependent TNF-α induced apoptotic cell death is seen in the 67NR cells. Here, we show a clear difference in tumor metastasis between the two cell lines and have shown that the TNF-α pathway could be responsible for the differences in metastatic potential.

Keywords: breast cancer, metastasis, apoptosis

Introduction

The Tumor Necrosis Factor (TNF) superfamily of cytokines consist of nineteen ligands [1]. All members of this family participate in pro-inflammatory activity through the activation of nuclear factor-κB (NF-κB) [1]. TNF-α is
the best studied and most cited members of the TNF family [1]. Tumor necrosis factor- alpha is a cytokine that was first discovered in the blood of mice injected with Bacilli Calmette-Guerin (BCG), a tuberculosis injection [2,3]. In the mid-1980s, the cDNA of TNF was cloned and recombinant TNF-α was made available [2,4].

The expression of this soluble factor is triggered upon activation of the immune system so that it can exert its cytotoxic effects in cells [4]. TNF is about 17-26 kDa and is expressed by activated immune cells and non-immune cells such as fibroblasts and endothelial cells [5-7]. Its transcription is regulated by NF-κB, c-Jun, activator protein-1 (AP1) and nuclear factor associated with activated T cells (NFAT) [5]. TNF can bind to two transmembrane receptor molecules: TNFR1 (also known as p55/p60) and TNFR2 (also known as p75/p80) [4, 5]. The trimeric TNF binds to the cysteine-rich repeats on the extracellular domains of the TNFR’s [4,8,9]. TNFR1 is expressed in most tissues while TNFR2 is typically only expressed in the immune system [4].

TNFR1 contains a death domain (DD) that is absent in TNFR2 [10]. The death domains play an important role in the assembly and activation of inflammatory and apoptotic complexes [11]. This domain has made TNFR1 an important protein in apoptotic signaling [10]. Upon trimeric TNF-α binding to TNFR1, TNFR1 trimerizes and silencer of death domain (SODD) is activated [10]. Further signaling ultimately results in the activation of endonucleases and DNA fragmentation, which are indicators of apoptosis [10]. For cell survival, TNFR1 activation could lead to the recruitment of TRAF-2, which initiates the phosphorylation of several proteins that results in the activation of cFos/Jun transcription factors through MAPK and JNK [10,12]. There is much debate about whether TNF prevents or promotes tumor growth. TNF has been shown to both promote apoptosis in cancer cells and stimulate cell survival, angiogenesis and metastasis [13,14]. Further characterization of TNF pathways will help clearly demonstrate the role that these proteins play in cancer.

4T1 mammary carcinoma cells are derived from a spontaneous tumor from a BALB/cfC3H mouse and are an excellent model for studying breast cancer in mice [15]. Upon injection into mice, the primary tumor grows quickly and metastasizes to the lungs, liver, bone, and brain [15,16]. Another cell line isolated from a BALB/cfC3H mouse is the 67NR line [17]. Though this tumor cell line is isolated from the same mouse type as 4T1 cells, it is non-metastatic [18]. Cell biology studies have revealed that 4T1 cells have an increase in cell adhesion, migration and invasion compared to 67NR cells [17]. Since these two cell lines provide a highly invasive and non-metastatic model of breast cancer, studying the differences in their gene regulation could provide some insight into the metastatic process.

To understand the gene differences in these cell lines, metastasis of disseminated 4T1 or 67NR cancer cells in BALB/c mice were investigated by systemic infusion of cancer cells by tail vein or after surgical removal of a primary tumor. Furthermore, 4T1 and 67NR cells were stably transfected with GFP and RFP plasmids so that the differences in tumor metastasis in different host tissue microenvironments could be quantitatively measured by culturing tumor cells in media. To gain insight into the specific molecular pathways between these two cell lines, differences in the expression of mRNA was measured using a mouse genome array. We show a clear difference in the tumor metastasis in BALB/c mice between the two cell lines and have shown that the TNF-α pathway could be responsible for the differences in metastatic potential.

**Materials and Methods**

**Cell lines and culture**

Murine breast tumor cell lines 4T1 and 67NR were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% fetal bovine serum (FBS), with antibiotics. Cells were incubated at 37°C in 5% (v/v) CO₂ and 95% (v/v) air mixture.
Cell viability assays

The viability and proliferation of 4T1 and 67NR cells was measured using an MTT assay. The tetrazolium compound used in the MTT assay is reduced by the mitochondrial dehydrogenase of metabolically active cells, thus conversion of the blue tetrazolium compound into a purple precipitate: formazan. This indicates the relative amount of viable cells. Quantification of formazen dye is measured using a colorimetric method. The 4T1 and 67NR cells were seeded in 24-well plates at a density of 2x10^4 cells/well in Dulbecco's Modified Eagle Medium with 10% FBS and allowed to adhere by incubating at 37°C for 24 h. After this step, cells were washed twice with PBS, and then 100 µL of MTT solution (MTT solution concentration is 5 mg/mL dissolved in PBS; thiazoyl blue tetrazolium bromide, catalog no. M5655; Sigma-Aldrich) and 900 µL of growth medium were added in each well. Cells were incubated at 37°C for 3 h, and solubilized with 1 mL of MTT solubilization buffer (anhydrous isopropanol containing 10% Triton X-100, 0.1 N HCL) for 5 minutes. Converted dye absorbance was measured in a spectrophotometer (DU-530 UV/VIS Life Science spectrophotometer; Beckman Coulter, Brea, CA) at a wavelength of 570 nm. The percentage of cell viability was determined by comparison with untreated controls.

PCR

Total RNA was extracted using TRIzol reagent according to the manufacturer’s instructions, followed by cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR for TNFRSF1A was performed using the following primers: 5'-GCTTCAACGGCACCGTGACAAT-3' forward primer and 5'CTGAGTCCGTGGGTTTGTGACATT-3' for the reverse primer. Quantitative Real-Time PCR experiments were performed in a LightCycler 480 instrument. Fold change in mRNA was calculated using 2^(-∆CT). ∆CT = CTgene - CTcontrol. β-Actin was used as the internal control.

Microarray

RNA was isolated from tissue culture dishes using the TRIzol reagent. 10µg of RNA was assessed on a mouse gene array chip (Agilent whole mouse genome 4x44 gene array, Catalog Number: G4122F).

Metastasis

Metastasis potential of 4T1 and 67NR cells in Balb/c mice was carried out after receiving approval from the Institutional Animal Care and Use Committee (IACUC), Tulane University Health Sciences Center. Four to six weeks old female Balb/c mice were obtained from Charles River Laboratories (Wilmington, MA) and maintained in a pathogen free environment at the Department of Comparative Medicine, Tulane University Health Sciences Center. The mice were handled and euthanized according to ethical guidelines. The mice were housed five per cage, fed ad libitum, and observed daily. The cages were kept in a climate controlled warm animal suite and cleaned on a weekly basis.

Metastasis of 4T1 and 67NR cells was performed using Balb/c mice. For each tumor cell line, mice were randomized into two groups wherein each group consists of five mice. One group of five mice was injected with 0.9% saline and used as a control. Another group of five mice was injected with either 4T1 or 67NR cells. The mice were closely monitored, weighed thrice weekly, and euthanized if they show any symptoms such as lethargy or body weight loss greater than 20% of initial body weight. Organs (lung, heart, liver, kidney, spleen, bone and brain) were collected for colony assay and histological evaluation.
Transwell Invasion Assay

The invasion assays were performed using Tanswell invasion chambers (Corning, Corning, NY) coated with 10 µg/ml Fibronectin on the bottom and matrigel on the top. 67NR and 4T1 cells were transferred to the top of the chamber with the indicated media. After incubation for 48 hours at 37°C in an atmosphere containing 5% CO₂, invaded cells on the lower surface were stained with crystal violet stain and counted under a light microscope.

H&E Staining

Animals were monitored for up to 50 days and then lung, heart, liver, bone, spleen, kidney and brain tissues were collected, fixed and embedded in paraffin for histological staining. For subcutaneous injections, mice were monitored for up to 72 days. Briefly, tissues were hydrated with decreasing concentrations of alcohol, and then rinsed with water. Samples were then stained with Hematoxylin followed by Eosin then mounted in mounting media.

Immunohistochemical characterization of breast cancer metastasis

At the end, mice were sacrificed; the individual organs (lung, heart, liver, spleen, kidney, bone, brain) were harvested, and fixed in 10% buffered formalin and embedded in paraffin. Paraffin blocks were prepared and five-micron tissue sections were prepared and stained with hematoxylin and eosin. Tissue sections were examined under a light microscope by two pathologists (Dr. Moroz and Dr. Jetly) for histopathological evaluation of tumor metastasis and photographed using a Nikon digital camera.

Quantitation of tumor metastasis with the colony assay

We used a previously described protocol to recover 4T1 and 67NR tumor cells from each organ that involves a combination of mechanical and enzymatic digestion [19]. Briefly, tissue was placed in a petri dish containing 10-ml of medium with heparin (5 units/ml) to maintain the viability of cells and prevent coagulation. The tumor cell isolation was performed as quickly as possible to maximize the viability of the tumor cells. Tissues were cut into small pieces of 1-3-mm³ and digested with collagenase type 1 (Invitrogen) for 30 minutes at 37°C. The cell suspension was filtered using a 22-micron nylon mess. Tumor cells were harvested by centrifugation at 500 rpm using a Beckman Table top centrifuge. The cell pellet was suspended and cultured in a growth medium containing 10% FBS, 1% penicillin and streptomycin and incubated at 37°C. The next day the culture was washed and replaced with a fresh growth medium. The cells were cultured for 2 weeks until visible cell colonies were obtained, and then colonies were stained using Giemsa dye (Sigma) and counted by a colony counter (Bio-Technologies Inc).

KM Plotter Online Survival Analysis

The relapse free survival of breast cancer patients was obtained from http://kmplot.com/breast [20].

Results

4T1 Tumors are more aggressive than 67NR

To determine the proliferation of 4T1 and 67NR cells, time lapse cell proliferation studies were performed. 4T1 and 67NR cells were counted every 24 hours for 96 hours and manually counted (Figure 1A). More 4T1 cells were present on the tissue culture dish for all time points. Cell proliferation was also determined with the MTT assay (Figure 1B). In both cases, 4T1 cells had a much greater rate of proliferation than 67NR cells.
Figure 1. Proliferation of 4T1 and 67NR Cells. Cell viability of 4T1 and 67NR cells was assessed by manually counting (A) and the MTT assay (B).

To evaluate tumor proliferation and dissemination in vivo, orthotopic 4T1 and 67NR breast cancer models were established in BALB/c mice. Tail vein injection of 4T1 cells into BALB/c mice resulted in a lower survival percentage than injection with 67NR cells (Figure 2A). The results from this experiment corroborate our in vitro findings which demonstrated that 4T1 cells display more aggressive behavior than 67NR cells. Histological evaluation of H&E stained lung, heart, liver, bone, spleen, kidney and brain from mice injected with the cell lines show an increase in staining of organs from 4T1 injected mice (Figure 2B). Quantitation of tumor cell dissemination with the colony assay revealed that the 4T1 cells developed metastases in the lungs, liver, heart, kidney, spleen, bone and brain while 67NR cells only metastasized to the lung (Figure 2C). These results demonstrate the metastatic potential of 4T1 cells.

About 40% of breast cancer patients will relapse after treatment of highly metastatic primary tumors [21]. To mimic this, subcutaneous injections were given to mice and tumors were extracted by surgical removal. Similar to the tail vein injections, mice injected with 4T1 cells resulted in a lower survival percentage than those injection with 67NR cells (Figure 3A). Metastasis was also observed after removal of the primary tumor. After primary tumor removal, histological evaluation of H&E stained lung, heart, liver, bone, spleen, kidney and brain from subcutaneous injections with the cell lines still showed an increase in staining of organs from 4T1 injected mice (Figure 3B). This time, quantitation of tumor cell dissemination with the colony assay revealed that the 4T1 cells developed metastases in the lungs, liver, and heart, while 67NR cells only metastasized to the lung (Figure 3C). These results suggest that even after removal of the tumor, mice injected with the 4T1 cells have a greater chance of relapsing.
Figure 2. Metastasis of 4T1 and 67NR Cells Injected by Tail Vein. A. Animal survival was monitored for up to 50 days after tail vein injection of 4T1 (n=5) and 67NR cells (n=5). B. H&E staining of lung, heart, liver, bone, spleen, kidney and brain tissues from tail vein injections with the 4T1 and 67NR cells. C. Quantitation of tumor cell dissemination with the colony assay from tissues of mice given tail vein injections.
Figure 3. Metastasis of 4T1 and 67NR Cells Injected Subcutaneously. A. Animal survival was monitored for up to 72 days after subcutaneous injection of 4T1 (n=5) and 67NR cells (n=5). B. H&E staining of lung, heart, liver, bone, spleen, kidney and brain tissues from subcutaneous injections with the 4T1 and 67NR cells. C. Quantitation of tumor cell dissemination with the colony assay from tissues of mice given a subcutaneous injection.
TNF-α pathways are down regulated in 4T1 cells

To gain insight into which molecular pathways differ between these two cell lines, differences in the expression of mRNA was measured using a mouse genome array. The genes up regulated in 67NR cells are the genes for TNFR/Fas, NIK, IKK, IκB, NF-κB, cIAP, Caspase 7, Acinus, Diablo, RTK, Ras, MEK1/2, ERK1/2, p90RSK, BAD, Bcl-2, and Bcl-XL (Figure 4A). The genes up regulated in 4T1 cells are the genes for Calpain, PLCγ, Caspase 3, ICAD, and Lamin A (Figure 4A). The 67NR cells have an up regulation of the TNF-α pathway and that could possibly be contributing to the inhibition of metastasis.

Figure 4. Characterization of the TNF-α pathway in 4T1 and 67NR cells. A. A gene array study confirmed that TNF-α dependent and independent apoptotic pathways are down regulated in 4T1 cells. B. Cell viability of TNF-α treated 4T1 and 67NR cells was assessed by manually counting. C. TNFR1 RNA expression was assessed in 4T1 and 67NR cells by RT-PCR. Expression of TNFR1 is greater in 67NR cells. Actin is used as a loading control. D. TNFR1
RNA expression was assessed in 4T1 (n=4) and 67NR (n=4) cells by qRT-PCR. Expression of TNFR1 is greater in 67NR cells. Actin was used as the internal control.

Next, we wanted to test cell viability after TNF-α treatment to see which cell type was sensitive to TNFR induced apoptosis. Figure 4B shows a dose dependent TNF-α induced apoptotic cell death in the 67NR cells. Just 10ng/ml is enough to reduce cell viability of the 67NR after 48 hours. TNF-α treatment of 4T1 cells showed no reduction in cell viability. Since 67NR cells are sensitive to treatment but 4T1 cells are not, we wanted to see whether the cells express TNFR1, the most abundant TNFR. RT-PCR of the RNA revealed that 67NR cells express TNFR1 but 4T1 cells do not (Figure 4C and 4D). This suggests that 4T1 cells are not responsive to TNF-α stimulation because they do not express TNFR.

Figure 5: TNF-α Pathway Inhibition with Thalidomide. A. Dose-dependent MTT assay of 67NR and 4T1 cells with 0-200 µg/ml Thalidomide. B. Time course MTT assay of 0 and 50 µg/ml Thalidomide. C. Transwell invasion assay with serum-free media in both the top and the bottom of the inserts, as well as, 0 and 50 µg/ml Thalidomide with complete media in the bottom of the insert. Number of cells per area was counted manually.
To determine whether the increased metastatic activity of 4T1 cells is specifically due to its lack of TNF-pathway proteins, we used the TNF inhibitor to assess cell proliferation and invasion. Thalidomide is a drug that promotes the degradation of TNF-α [22-24]. Concentrations of Thalidomide as low as 10 µg/ml are enough to see a reduction in the expression of NF-κB in some cell lines [25,26]. Treatment with Thalidomide did not greatly affect the proliferation of 67NR cells, but it decreased the proliferation of 4T1 cells (Figure 5A). Furthermore, at 72 hours, treatment of 4T1 cells with just 50 µg/ml of Thalidomide was enough to reduce cell proliferation (Figure 5B) suggesting that Thalidomide can regulate cell proliferation by affecting NF-κB and this effect is possibly independent of TNF-α functions. Next, we examined the effect on cell invasion, a surrogate in vitro assay for metastasis. Treatment of 67NR and 4T1 cells with Thalidomide increased the number of 67NR cells invaded, while having little effect on the number of 4T1 cells invading (Figure 5C). These results suggest that inhibition of TNF-α signaling pathway promotes cell invasion in 67NR cells and that the differences in the metastatic capacities of 67NR and 4T1 cells are likely due to alterations in the TNF-α pathway.

To assess TNF-α expression in human breast cancer tissues, we queried the kmplotter database. Patients with high TNFA, TNFR1, and TNFR2 expression have a higher probability of relapse free survival (Figure 6A-6C) [27]. This suggests that this TNF pathway does prevent metastasis.

**Figure 6. Relapse-Free Survival of Human Breast Cancer Patients.** Probability of relapse free survival in patients with high or low expression of TNF (A), TNFR1 (B), and TNFR2 (C).

**Discussion**

The goal of this study was to identify the differences in the molecular pathway between high and low metastatic murine mammary cancer cells by using a gene array. 4T1 and 67NR mammary carcinoma cells are derived from spontaneous tumors from BALB/cfC3H mice [15]. Orthotopic 4T1 and 67NR breast cancer models established in BALB/c mice were used to evaluate tumor proliferation, dissemination and animal survival. 4T1 cells were able to disseminate into multiple organs and our results demonstrate the metastatic potential of 4T1 cells. We next wanted to look at metastasis after the removal of the primary tumor. We observed the same aggression in the 4T1 cells and the results suggest that even after removal of the tumor, mice injected with the 4T1 cells have a greater chance of relapsing.
To gain insight into the specific molecular pathways between these two cell lines, differences in the expression of mRNAs was measured using an oligonucleotide array. We found that the 67NR cells have an up regulation of the TNF-α pathway that could possibly be contributing to the inhibition of metastasis. There has been much controversy surrounding whether TNF-α is a tumor suppressor or oncogene. TNF has been shown to promote apoptosis in cancer cells but it has also been shown to stimulate cell survival, angiogenesis and metastasis [13,14]. Stimulation of TNF-α showed a dose dependent reduction in the viability of 67NR cells, but not 4T1 cells. TNFR1 contains a death domain (DD) that leads to apoptosis upon activation [10]. Next we wanted to know whether TNFR1 could be causing the reduction in cell viability of 67NR cells. RT-PCR revealed that 67NR cells express TNFR1 but 4T1 cells do not. This explains that 4T1 cells are not responsive to TNF-α stimulation because they do not express TNFR.

Furthermore, by using the TNF-α inhibitor, Thalidomide, we have determined that the differences in the invasive capacities of 67NR and 4T1 cells are specifically due to alterations in the TNF-α pathway. Addition of recombinant TNF-α did not alter 4T1 viability, but decreased 67NR viability. Alternatively, addition of the TNF-α inhibitor no longer decreased the viability of 67NR cells. These data suggest that TNF-α signaling plays an important role in metastasis. Patients with high TNFA, TNFR1, and TNFR2 expression have a higher probability of relapse free survival [27]. TNF plays a role in many cellular events, including cell survival, differentiation, proliferation and death [13]. Upon trimeric TNF-α binding to TNFR1, TNFR1 trimerizes and silencer of death domain (SODD) is activated [10]. Further signaling ultimately results in the activation of endonucleases and DNA fragmentation, which are indicators of apoptosis [10]. Our results show a clear difference in the tumor metastasis in BALB/c mice between the two cell lines and have shown that the TNF-α pathway could be responsible for the differences in metastatic potential. Since these two cell lines provide a highly invasive and non-metastatic model of breast cancer, studying the differences in their gene regulation could provide more insight into the metastatic process.

References