Advances in Clinically Relevant Metastatic Breast Cancer Models

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Despite advances in surgical and clinical management, metastatic disease, notably to the lungs, liver, bone, and brain is the most common cause of death from breast cancer. Two basic principles govern the process of metastasis. First, that the tumors are heterogeneous populations of cells, and second, that the process of metastasis is a sequence of events that depends on tumor cell properties and interactions with the microenvironment at the sites of metastasis. In theory, inhibitors targeted at any of the steps of metastasis have the potential to inhibit metastatic progression. *In vitro* assays cannot simulate accurately the complex process of metastasis, and the use of appropriate animal model is necessary to model the process, and to test the impact of targeted inhibitors on the growth and development of breast cancer metastasis. Animal models for growth and metastasis of rodent and human breast cancer cells have been developed, including models that target the metastatic growth in key organs such as the bone and brain. **(Journal of Korean Breast Cancer Society 2004;7: 141-147)**

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INTRODUCTION

Once breast cancer has been diagnosed, the most important question is whether the cancer is confined to the breast or has spread to distant sites. The majority of the death of women with breast cancer result from the growth of metastases that do not respond to therapy.(*1*) The development of more effective therapies should be based on a better understanding of the mechanisms responsible for the spread of cells from the breast to distant sites, including lymph nodes, bone, brain, liver and lungs. A variety of *in vitro* and in vivo models have been developed to study the biology of metastasis.(*2*) In general, *in vitro* assays have been designed to model distinct steps in the process, for example, invasion through the reconstituted basement membranes,(*3*) or specific binding to endothelial cells isolated from organs where the cancer cells commonly form metastases.(*4,5*) Such *in vitro* assays have great practical value for evaluating specific tumor cell behaviors, yet their limitations for predicting in vivo malignancy should always be considered. It is probably impossible to simulate accurately all the events of the metastatic process with *in vitro* models, especially considering the events that involve interactions with components of the microenvironment at the site of metastasis.(*6*) Thus, animal models using transplantable tumors that can grow and metastasize predictably in a suitable host have become standard systems for analyzing the metastatic phenotype and testing the efficacy of anti-metastatic therapies. The most common animal models are rodent tumor models, using transplantable tumors, or spontaneously arising or carcinogen- induced mammary tumors of rats and mice.(*7*) More recently, transgenic mice with different oncogenes targeted to the mammary epithelium have become available and some are suitable for testing specific forms of therapy, such as those designed for tumors that overexpress HER2/neu.(*8,9*) Immunodeficient rodents, most commonly athymic (also known as nude) of SCID mice, have been used widely for xenograft studies with human cancers. Not all human cancers or established tumor cell lines will successfully grow in immunodeficient mice, at least from a subcutaneous (s.c.) route of inoculation, the most common and for practical purposes the easiest technique to use. The approach of injecting human tumor cells into the normal equivalent mouse organ, known as orthotopic injection, has been adopted as a way to improve tumor take and growth, and has also been shown to increase

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the likelihood of metastasis.(*10*) For breast cancer cells the appropriate site for implantation is the mammary fatpad (mfp) and there is extensive literature describing growth-modulating effects of the mfp on normal, preneoplastic and malignant mammary epithelial cells.(*11,12*) Injection of human breast cancer cells into the mpf has been shown to result in improved tumor take and growth rates compared with s.c. injection, and has allowed the selection of more metastatic variants of human breast cancer cells, by isolating cells from the metastatic lesions in the immunodeficient mice.(*13,14*) However, while mfp injection generally improves tumor takes and growth rates, the number of cell lines that reproducibly form spontaneous metastases is disappointingly low, especially since the majority of the commonly used breast cancer cell lines were established from metastases.(*2*) The orthotopic xenotransplantation of histologically intact fragments of human cancers, such as tumor specimens taken directly from the patient, can result in enhanced reproduction of the metastatic potential of the cancer cells.(*15*) An explanation for this may be that the stromal elements in the tissue fragments allow continued expression of genes essential for malignant growth and metastasis. In contrast, when the tumor cells are separated from stroma and grown in tissue culture, the turmor-stroma interactions are lost and metastasis promoting gene expression may be reduced or silenced. However, while the use of histologically intact tissue xenografts has proven advantages, one disadvantage is that of limited tissue availability compared with the use of established cancer cell lines. These, at least in theory, provide consistent and reproducible models in different laboratories.

Clinical observations suggest that the responses of breast cancer metastases to chemotherapy can be influenced by the anatomical location of the lesions, possibly related to the differences in microenvironmental stress.(*16*) Differential chemo-sensitivity of metastases in different organs may be a function of heterogeneity of the tumor population, with different clones metastasizing to different organs. However, the influence of the organ microenvironment cannot be ignored. Results from experimental tumor models have shown that the same tumor implanted in different organs can have different responses to a chemotherapeutic drug.(*17*) For example, sensitivitity of mouse mammary tumor cells to different chemotherapeutic agents was assessed in vivo, comparing responses in s.c. tumors with response of the cells in bone marrow, spleen, liver, lungs and brain. The s.c. tumors were generally sensitive, while cells in liver and brain were less sensitive to alkylating agents. Cells in bone marrow showed variable sensitivity to different agents, and addition of an anti-angiogenic compound markedly increased killing of bone marrow micrometastases by cyclophosphamide.(*18*) Thus the tissue microenvironment may contribute to the sensitivity of metastatic cells to chemotherapy, and modulating the stroma (in this case by inhibiting angiogenesis) can have an impact on the response to treatment.

Experimental models have been developed that can target tumor cells to different organs to simulate metastasis in specific sites, and therefore be used for studying response of tumor cells growing in different organ environments. Intravenous (i.v.) injection of tumor cells into the tail vein of mice usually results in lung metastasis, and injection into the spleen or the hepatic portal vein can generate experimental metastases in the liver.(*19,20*) Experimental models of brain metastasis have use direct injection into the brain parenchyma, or introduction of cells into the internal carotid artery.(*21*) Injection of tumor cells into the left heart (intra-cardiac, i.c.) leads to widespread distribution of metastases, including bone and bone marrow.(*22*) This model has been used successfully as a model for osteolytic bone metastasis using the MDA-MB-231 human breast cancer cell line.(*23*) Direct injection of breast cancer cells into the bone (e.g. tibia or femur) can result in localized tumors.(*24,25*) While the direct injection approach cannot be used to investigate events in the initial steps of bone metastasis, it can be used for studies of interactions between tumor cells and the bone microenvironment. Spontaneous bone metastasis by human breast cancer cells is rarely seen,(*26*) but a mouse mammary tumor model, 4T1, syngeneic to BALB/c, is reported to form bone metastases from mfp tumors. Clones of the original tumor were isolated and characterized with increased bone-metastiasizing ability.(*27*) This, and another studies using human tumor cell lines, are examples of using an animal model to isolate variants with increased metastatic ability, in some cases for metastasis to a specific site.(*14,28*) Such variants can then be used for further phenotypic characterization, and also for pre-clinical therapy models.

This chapter describes three techniques for metastasis models using human breast cancer cells; mammary fatpad injection, intra-cardiac injection and intra-carotid injection.

MATERIALS

1) Human tumor cell lines, free of mycoplasma and murine pathogenic viruses (retrovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus,

mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, lactate dehydrogenase virus). Checking the cell lines for these viruses will reduce the risk of introducing pathogens into the animal facility.

2) Nude mice, 6 weeks old at the start of experiment.

3) Culture medium with serum.

4) PBS without Ca^{2+} and Mg^{2+}

5) Trypsin-EDTA: 0.25% w/v trypsin and 0.02% w/v EDTA in PBS without Ca^{2+} and Mg^{2+}. Prepare a fresh trypsin solution before harvesting cell cultures.

6) Sterile instruments and surgical supplies for necropsy and surgery (forceps, scissors, sutures and sterile cotton tip (Q-tip) for intra-carotid artery injection and 12 mm wound clips and wound clip applier).

7) Alcohol wipes and Betadine scrub, or equivalent antiseptic scrub solution.

8) Mouse restraint device for intra-carotid artery injections (small board and rubber bands)

9) Sterile 1 ml tuberculin syringes and 27 gauge (G) × 1/2" (13 mm) needles; plastic cannula prepared from a disposable tuberculin syringe for intra-carotid artery injections.

10) Anesthesia; either i.p. (intraperitoneal) injection of Nembutal (sodium pentobarbital) 50 mg/kg in PBS, or an inhalation anesthetic such as Metofane (Methoxyflurane)

11) Dissecting microscope.

12) Warming lamp or pad.

13) Calipers for tumor measurements.

14) 10% neutral buffered formalin.

Two critical elements for working with immunodeficient mice are the facility in which they are housed, and the areas used for experimental manipulations. Ideally the animals should be housed in a Specific Pathogen Free (SPF) Barrier Facility, in microisolator cages. All manipulations should be performed in laminar airflow workstations, or an area that is designated solely for working with immunodeficient animals. Depending on the facility, working with the immunodeficient mice may require changing into surgical scrubs, sterile coveralls, caps, masks, shoe covers and gloves. Work patterns must be organized such that working with and monitoring immunodeficient animals precedes any work with immunocompetent mice in the same day.

METHODS

1) Preparation of cells for injection

(1) Aspirate culture medium from culture of tumor cells that are between 75 and 90% confluent (plate cells, or add fresh medium the previous day to obtain actively growing cultures) (See Note 1). Wash with 10 ml of PBS per 75-cm2 flask; add 1～2 ml of the trypsin-EDTA solution. Incubate 30s-1min., then agitate, shake, or tap the flask in the palm of one hand to detach the cells.

(2) Resuspend the cells in 10 ml of culture medium and transfer to a centrifuge tube. Spin at 200-x g for 10 min., and resuspend the pellet in PBS.

(3) Determine cell number, and adjust the concentration for the appropriate inoculum volume, by centrifugation and resuspension in a smaller volume of PBS.

(4) Place the suspension in ice and proceed immediately to inject the cells.

2) Mammary fatpad injection

(1) Anesthetize a female mouse, lay it on one side, and clean the skin of the opposite side in preparation for surgery. Make a 5-mm incision in the skin over the lower lateral thorax. Open a pocket under the skin in a cranial direction with the scissors, so that the mammary fatpad can be seen.

(2) Vortex the cell suspension and draw it up into a 1ml syringe. Place a 27-G needle on the syringe and expel any air bubbles.

(3) Insert the needle into the fatty tissue of the mammary fatpad, and inject 0.1 ml with 2×10^6 cells for the MDA-MB-435 human breast cancer cell line (See Note 2). The inoculum should form a bubble inside the fatpad, and not leak into the s.c. space. Close the incision with wound clips, and monitor the mouse until recovered from the anesthesia. Return the mouse to a clean cage.

(4) Monitor mice daily for overall condition, and measure tumor growth once or twice weekly (See Note 3). Hold the mouse by grasping it firmly in one hand. Secure the scruff of the mouse neck between thumb and forefinger with the tail between the third and fourth fingers and the palm of the hand and use calipers to measure two diameters of the tumor. Calculate the mean diameter to graph out tumor growth over time. The diameter measurements can also be used to estimate tumor volume, using the formula:

Tumor volume= $x^2y / 2$

Where x is the smaller diameter of the tumor and y is the larger

(5) When the tumor reaches a maximum size of 1.5 cm, either kill the mouse or remove the tumor. The MDA-MB-435 cell line can form tumors of this size in 10 to 12 weeks. If the tumors are removed, kill mice 4～6 weeks later.

(6) Euthanize mice and examine for metastases, principally

in lungs and lymph nodes, but examine the abdomen also. Fix organs or tissues in formalin and prepare sections for histology if required. If a mouse had been showing abnormal balance or movements, remove the brain for histology (See Note 4).

3) Intra-cardiac injection

(1) Anesthetize a mouse, place on its back on a clean work surface, and clean the skin over the chest.

(2) Vortex the cell suspension and draw 0.1～0.2 ml into a tuberculin syringe with a 27 G needle, leaving a small bubble of air at the below of the syringe plunger. Insert the needle into the second intercostal space, 3-mm to the left of the sternum, directing into the center of the chest, to a depth of 6 mm. Pulsatile flow of red blood into the hub of the needle will indicate correct placement of the needle in the left ventricle, and gentle aspiration may be needed if red blood does not appear immediately.

(3) Slowly inject 0.05～0.1 ml of suspension over 20～30 sec. Do not inject the air bubble.

(4) Withdraw the needle, place the mouse on its side and allow it to recover from the procedure, with supplemental heat if necessary. Return the mouse to a clean cage after it has completely recovered from the anesthetic.

(5) Observe the mice daily for signs of tumor burden, including paralysis, hunched posture, or weight loss. Euthanize when moribund or at pre-determined time points, and necropsy and preserve tissue for histology if required. Examination of the skeleton by radiography can detect skeletal lesions. The incidence and distribution of metastases may vary for different tumor cell lines (See Note 5).

4) Intra-carotid artery injection

(1) Prepare a plastic cannula by melting and stretching the hub of a 1-ml disposable syringe, to create a ＜30 G cannula.

(2) Anesthetize a mouse with i.p. injection of sodium pentobarbital (50 mg/kg) and restrain on its back on a clean board. Stabilize the head by placing a rubber band under the upper incisors and around the board.

(3) Clean the neck of the mouse with alcohol and Betadine.

(4) Cut the skin of the neck with a mid-line incision, and place the mouse under a dissecting microscope. With blunt dissection, expose the trachea and muscles to expose the right common carotid artery (See Note 6).

(5) Prepare the artery distal to the point of bifurcation of the internal and external carotid arteries.

(6) Place and tie a ligature of 5-0 black silk suture in the common carotid artery, proximal to the point of injection.

(7) Place and loosely tie a second ligature at the point of bifurcation of the internal and external carotid arteries. Place a sterile cotton tip under the artery just distal to the injection site.

(8) Vortex the prepared cell suspension at the required concentration (1×10^5 cell in 0.1 ml) and fill the syringe.

(9) Nick the common carotid artery with microscissors between the ligatures. Lifting the cotton tip can control backflow bleeding from distal vessels. Insert the plastic cannula through the hole into the vessel lumen and thread forward into the internal carotid artery. Slowly inject the cell suspension, withdraw the cannula and tighten the second ligature.

(10) Close the skin incision with clips or sutures. Allow the mouse to recover, using a heat lamp if necessary, and when it is fully mobile place it in a clean cage.

(11) Inspect the mice daily, watching for signs of tumor growth including the development of paralysis, abnormal movements, swelling of the skull, and weight loss. Typically, the survival time of mice injected with 1×10^5 MDA-MB-231 human breast cancer cells was 50 to 60 days, killing mice that displayed these signs and not using death as the endpoint.(*29*)

NOTES

1) Preparation of cell suspensions: Some of the variability in repeated experiments with a particular cell line, or from published results from other laboratories may arise from inconsistencies in techniques or poor quality preparation of the cells for injections. To optimize the results and consistency between experiments, thaw a vial from frozen stocks of the cell line and expand the cells in tissue culture to obtain the required cell number. The cell should be in sub-confluent, actively growing cultures. The cells from confluent cultures are more likely to form clumps of aggregates, depending on the cell type. In addition, the degree of confluence *in vitro* has been reported to regulate gene expression, which might impact on the *in vivo* behavior. The important point is to be cosistent in using good cell preparation techniques. High viability is essential. The method described generally yields cell suspensions with high viability (98～100%, by Trypan blue dye exclusion). If a suspension has less than 90% viability, or if the cells are in clumps, it would be best to discard these cells and start with a fresh culture. For experi-

mental metastasis assays using intra-vascular injection, clumps of cells, or dead cells mixed with live tumor cells might artificially increase the number of tumor colonies formed. Using the Ca^{2+} and Mg^{2+}-free buffer will retard formation of clumps, and gentle vortexing may help to break up loose clusters, but if cells come off the tissue culture flask in clumps it is best to start with fresh, less confluent cultures. Too vigorous pipetting or mixing may be more likely to damage the cells than break up the clumps. As stated above, once the suspension has been prepared, proceed to inject as soon as possible. Keeping the suspension on ice will reduce the formation of cell aggregates.

2) Breast cancer models: The methods described uses an estrogen-receptor (ER) negative breast cancer cell line. Cell lines that express ER may not grow unless the nude mice are supplemented with estrogen. One commonly used method is the implantation of slow release pellets of 17β-estradiol (from innovative Research of America, Sarasota, Florida, USA). A 60-day release 0.72-mg pellet will support the growth of the ER-positive MCF-7 breast cancer cell line (from injection of 5×10^6 cells into the mammary fatpad)

3) All of the animal procedures (housing conditions, experimentation, surgical procedures, euthanasia and anesthesia, etc.) will probably be regulated by an institutional body such as an Institutional Animal Care and Use Committee. In the U.S.A. this committee is charged with ensuring compliance with guidelines and requirements established by the Public health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act of 1966 as amended. Experimental design should take into account the well being of the mice and use appropriate procedures to reduce pain and suffering. In the context of this chapter this means careful monitoring of mice for development of tumor burden, appropriate animal handling and surgical procedures, and the humane use of euthanasia. Using a moribund end-point, rather than a death endpoint for a study is more practical if the point of the study is to assess the extent of tumor spread. Recognizing the signs of tumor development comes with experience, and regular monitoring of the condition of the animals. Autolysis of mouse tissues starts rapidly, and it is easier to monitor, measure and recover metastases from freshly killed mice than from those dead for more than an hour or two. Furthermore, if tissues are needed for analysis such as nucleic acid extraction or immunohistochemistry, these should be harvested immediately after killing the mouse. If a veterinary medicine department is administering the animal facility, this is a source for advice on small animal surgery, anesthesia, euthanasia and necropsy techniques. Inhalation of Metofane is a rapid and easy means of anesthesia, and is ideal for short procedures, as the mice will recover rapidly. Metofane is a hazardous agent, and should therefore only be used in a suitable fume hood or with appropriate ventilation. It is also not currently manufactured in the U.S.A. Alternative inhalant anesthetics, such as isoflurane and halothane require specialized vaporizer apparatus. Injectable anesthetics such as Nembutal (sodium pentobarbital, 50 mg/kg injected i.p.) have longer induction and recovery times. When using anesthesia on nude mice take precautions to prevent hypothermia, and do not be too liberal with alcohol and surgical scrub fluids. Use a warming pad or lamp during the recovery phase, but do not let the mouse overheat either.

4) Scoring the metastases: The simplest method is to count the numbers of metastases visible on the surface of the target organs. An alternative to aid detection of metastases is to fix the organs in Bouin's fixative. The metastases will be white lesions against the yellow stained normal tissue. Counting surface lesions does not include microscopic disease which can be detected in histological sections, although quantitation of metastases in multiple organ sections is labor-intensive. Depending on the model used and the site of metastasis, the weights or volumes of organs can be used to estimate the tumor burden (discussed in reference 20). The use of fluorescent markers, notably the green fluorescent protein (GFP) (*30*) can facilitate the detection of cells and micrometastases. Another technique that requires transfection of the tumor cells is introduction of a luciferase gene. When animals with luciferase-expressing tumors are injected with the substrate luciferin, the resulting chemoluminescence can be measured non-invasively, and is proportional to the tumor burden.(*26,31*) Both techniques require specialized equipment for detecting and measuring the fluorescence or luminescence. How the metastatic burden is measured will dictate the choice of test used for statistical analysis. For the comparisons of numbers of metastases estimated by surface counting, use a non-parametric test such as the Mann-Whitney rank sum test. Consider the analysis to be used when planning the study, to ensure that there are enough animals per experimental group to achieve statistical significance. If possible, allow for the loss of one or two mice (from early morbidity, or reasons unrelated to the experiment) and still have enough data points for valid statistical analyses.

5) The distribution pattern and the length of time before metastases develop may differ for each cell line, and may also differ from what has been published for a particular cell line. In the first experiment with a cell line, monitor the mice closely and, if necessary, wait longer than expected for the mice to show signs of metastatic tumor burden. Killing mice at different time points can also be done to monitor and establish the time course of growth of metastases (assessed macroscopically or in histological sections). No or fewer metastases than expected could be the result of a number of factors, including the health and housing conditions of the mice, and the cell preparation techniques. Variants of some human tumor cell lines have arisen, possibly resulting from different tissue culture techniques, which vary considerably in their tumorigenic and metastatic phenotypes. To save time and resources, it may be prudent to obtain a particular cell line from an investigator who is currently using the cells for in vivo studies.

6) Intra-carotid artery injection is a challenging technique. Previous experience with microsurgery is an advantage, and taking time to practice the technique is probably essential. An assistant who can monitor the level of anesthesia in the mice, and hand supplies or instruments to the operator would also be helpful. Close post-procedure monitoring of the injected mice is recommended. If there is high mortality in the first few days after injection, the dose or volume of the inoculum may need to be reduced in subsequent experiments.

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