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Review

Modeling human breast cancer metastasis in mice: maspin as a paradigm

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Summary. Breast cancer is the most common cancer detected in women, accounting for nearly one out of every three cancers diagnosed in the United States. Most cancer patients do not die from the primary tumor but die due to metastasis. Therefore, the study of metastasis is of most importance both to the clinician and patient. In the past, animal models have been used in breast cancer research and mammary gland biology. Our group has also established several animal models to address the function of a novel tumor suppressor gene maspin in breast tumor progression. Maspin was initially isolated from normal mammary epithelial cells. Its expression was down regulated in breast tumors. To test the protective role of maspin overexpression in mammary tumor progression, we crossed maspin overexpression transgenic mice (WAP-maspin) with a strain of oncogenic WAP-SV40 T antigen mice. The bitransgenic mice had reduced tumor growth rate and metastasis. Maspin overexpression increased the rate of apoptosis of both preneoplastic and carcinomatous mammary epithelial cells. Maspin reduced tumor growth through a combination of reduced angiogenesis and increased apoptosis. In a separate animal experiment, maspin overexpressing mammary tumor cells (TM40D) were implanted into the fat pad of syngeneic mice. TM40D tumor cells were very invasive and metastatic. However, both primary tumor growth and metastasis were significantly blocked in TM40D cells that overexpress maspin as a consequence of plasmid or retrovirus infection. These evidences demonstrate that maspin function to inhibit primary tumor growth as well as invasion and metastasis. Elucidating the molecular mechanism of maspin action will shed light on our understanding of breast cancer invasion and metastasis.

Key words: Maspin, Mammary tumor progression, Invasion and metastasis, Breast cancer model

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Introduction

Breast cancer is the most common cancer detected in women, accounting for nearly one out of every three cancers diagnosed in the United States (Howe et al., 2001). In 2001 alone, approximately 192,000 women were diagnosed with this disease. Only lung cancer causes more cancer-related deaths among women (Howe et al., 2001). In the past, the study of breast cancer has focused primarily on tumor initiation and suppression of primary tumor growth, with less effort devoted to understanding the process of tumor metastasis and invasion (Weidner et al., 1991; Welch et al., 2000; Folkman, 2001). Nevertheless, metastasis is of most importance both to the clinician and patient. Currently, chemotherapy, radiation therapy and surgery are used to treat breast cancer in patients (Stephens et al., 1992; Fisher, 1996). The single most effective therapy for those diagnosed with breast cancer is the removal of the primary tumor by surgery. However, residual tumor cells that metastasize to secondary sites, such as the lung and bone, is the ultimate threat to every patient following surgery (Folkman, 1985; Fidler and Ellis, 1994). It is clear that most cancer patients do not die from the primary tumor but die due to metastasis.

Metastasis is an extremely complicated process involving local invasion, intravasation and extravasation of vascular system, growth at secondary site and angiogenesis (Liotta et al., 1983a, 1991; Stetler-Stevenson, 1993; Fidler and Ellis, 1994). To address tumor invasion and metastasis one must establish an appropriate animal model in which the tumor cells are invasive and can metastasize to other organs. The animals used better have an intact immune system so that the process of metastasis can mimic what occurs in human patients. Historically, animal models have played an important role in breast cancer research and mammary gland biology. Before the age of genetic engineering, chemical carcinogens were widely used to induce mammary tumors in rodents. Some of the most common carcinogens used were 2-AAF(2-acetylaminofluorine), 3MC (3-methylcholathrence), and DMBA (dimethylbenzanthracene) (Medina, 1974a;

Reigh et al., 1978). In mice, hormone levels of animal were found to be important in stimulating mammary tumorigenesis (Medina, 1974b). Progesterone and pituitary isografts, which stimulate the level of estrogen, progesterone and prolactin, were found to be key factors for promoting carcinogen-induced tumorigenesis. These early studies provided valuable information about mammary tumorigenesis and the shift from hormone dependent to independent growth. In addition, people noticed that some mice developed mammary tumors spontaneously (Gruntenko et al., 1972). Initially, it was thought that the cause of such cancer was due to the "milk factor" because the cancer was transferred from the mother of the high incidence strain to the offspring (Gillette, 1976). This milk factor was later discovered to be an RNA containing virus (mouse mammary tumor virus, MMTV). The study of MMTV-induced tumors and the use of genetic engineering over the last two decades have dramatically changed the study of mammary tumors. Presently, use of the promoter (LTR) from MMTV to target transgene overexpression or for specific gene deletion, has generated more than one hundred mouse models for studying mammary cancer progression (Schmidt et al., 1988; Pattengale et al., 1989; Cardiff et al., 2000; Hennighausen, 2000).

Despite the existence of this large collection of mammary tumor models, few have been characterized for the mechanism of invasion and metastasis. This does not mean that mouse mammary tumors are less likely to metastasize. As evident from the studies from our laboratory and other investigators, tumor metastasis occurs at high frequency in these tumor-bearing mice (Guy et al., 1992; Cardiff et al., 2000; Mundy, 2001). We hypothesize that by allowing tumors to grow larger or by removing the primary tumors so that the animals can be kept alive for longer periods of observation, one should be able to detect mammary tumor metastasis in a large percent of animal models.

Maspin as a paradigm for metastasis characterization

Our interest in cancer metastasis began with the characterization of the maspin gene function in mammary tumors. The maspin gene was initially cloned by differential screening of normal human mammary epithelial cells and breast tumors. Maspin was expressed at the mRNA level in normal but not in tumor-derived human mammary epithelial cells (Zou et al., 1994). Human maspin is encoded by a cDNA of 2584 nucleotides that produces a 42 kDa protein with overall sequence homology to serine protease inhibitors, or serpins. Thus, the name maspin was derived from the mammary homologue to serpins. To characterize the function of maspin, recombinant maspin proteins were produced in *E. coli* and insect Sf9 cells. Purified maspin was a potent inhibitor to invasion and motility of mammary tumors as well as other cell types (Sheng et al., 1994, 1996; Zhang et al., 1997). Morphologically, maspin treatment led to a partial restoration of the benign epithelial morphology and an increased cell adhesion to fibronectin (Sheng et al., 1996; Seftor et al., 1998). Time-lapse video microscopic studies showed that recombinant maspin dramatically inhibited the lamellopodia extension and vectorial translation, but did not inhibit the membrane ruffling activities of the cells.

Initially, investigators reported that maspin did not undergo typical transition from the stressed to relaxed state. Thus it was predicted that maspin might act as a non-inhibitory serpin (Pemberton et al., 1995). However, other investigators have shown that maspin could interact with tPA in the presence of fibrinogen and poly-L-lysine (Sheng et al., 1998). Recently, using a yeast two-hybrid approach, maspin was shown to interact with extracellular matrix collagen I and III (Blacque and Worrall, 2002). We believe that maspin is likely to interact with multiple target molecules, such as its homologue PAI-1 that also functions through interactions with several different proteins (Deng et al., 1996; Stefansson et al., 1996).

Over the last few years, we have focused our efforts to characterize the role of maspin in breast tumor progression, demonstrating that maspin is indeed capable of inhibiting tumor growth as well as tumor invasion and metastasis. In this review, we summarize our utilization and characterization of several mammary tumor metastasis models with maspin as the paradigm.

WAP-SV40 T antigen as a model for the study of mammary tumor progression

To characterize the function of maspin during tumor progression, we generated transgenic mice containing the mammary specific whey acidic protein (WAP) promoter to overexpress maspin specifically in the mammary epithelial cells (Zhang et al., 1999). Overexpression of maspin in normal mammary epithelial cells inhibits mammary gland development and induces apoptosis. In order to test the protective role of maspin overexpression in mammary tumor progression, we crossed these WAP-maspin transgenic mice with a strain of oncogenic WAP-Simian Virus (SV) 40 T antigen (TAg) mice. WAP-TAg transgenic mice develop mammary tumors with 100% frequency and can be utilized to examine specific mechanisms of tumor progression at both early and late time points (Tzeng et al., 1993; Li et al., 1996). The SV40 TAg initiates tumorigenesis through the inactivation of both the p53 and the pRb related family of proteins (Dyson et al., 1989; Li et al., 2000). In human breast cancers inactivation of p53 function is found in nearly 40% of tumors, and mutations in Rb or related proteins have also been reported (Lee et al., 1988; Li et al., 2000).

In preneoplastic mammary cells, we found that maspin overexpression in bitransgenic mice increased the rate of apoptosis. Similarly, during tumor progression overexpression of maspin reduced tumor growth through increased apoptosis in primary

adenocarcinomas. This was demonstrated by comparing the rate of tumor growth of the WAP-TAg and the bitransgenic mice. Tumors were measured biweekly after initial detection, and the mice were euthanized when the primary tumor grew to 2.5 cm in diameter. To assess the rate of tumor growth, measurements were taken from the appearance of the first palpable tumor to the time when the tumor grew to 2.5 cm in diameter. The tumor growth rate in mice overexpressing maspin significantly decreased from 36.3 to 49.3 days ($p< 0.03$). To determine if maspin overexpression had an effect on microvessel density and apoptosis during tumor progression in vivo, microvessel density in adenocarcinomas with and without maspin overexpression were compared. Microvessel density was measured after CD31 staining. In small mammary tumors (tumor size ≤ 0.6 cm in diameter), the microvessel density was significantly reduced ($p<0.02$), while the apoptotic index was significantly increased in the presence of maspin overexpression ($p<0.01$).

To determine the effect of maspin overexpression on lung tumor metastasis, lung tissues were serially sectioned for microscopic analysis. When the primary tumors grew to approximately 2.5 cm in diameter, all mice were sacrificed. The bitransgenic mice had a reduced rate of metastasis compared to that of the WAP-TAg single transgenic mice. Lung metastases developed in fifteen of the twenty-six (55.6%) WAP-TAg mice, while in bitransgenic mice the rate of metastasis was 37.5% (fifteen of the forty bitransgenic mice). To compare the difference in the number of tumor foci between these two mouse strains, the microscopic images of lung sections were captured and the number of foci per area of the lung was quantified for each mouse. The bitransgenic mice had decreased foci numbers ($0.356/10^4$ pixels) compared to that of WAP-TAg mice ($0.655/10^4$ pixels).

The strength of the SV40 TAg model is that mammary tumors develop with 100% frequency within 6 months after the first pregnancy, which activates the SV40 TAg oncogene. Both SV40 TAg and bitransgenic mice required mating with male mice throughout the study to continually activate transgene expression. Tumor development follows a defined pattern from hyperplasia to well- and poorly differentiated morphologies. In the background of NMR and C57BL/6, we analyzed 66 transgenic mice that developed an average of three mammary tumors per animal at different sites in the mammary gland. When the primary tumors were grown to about 10% of body weight, we observed that 56% of animals had tumor metastases to the lung. Histology of the lungs showed that in addition to the true metastatic foci, there were many tumor cells localized within the vessels of lung. This kind of tumor foci is not considered a metastasis but is termed a tumor embolus. The ability of SV40 TAg tumors to grow within the micro-vessels is interesting and may reflect the ability of these tumor cells to adhere within the endothelium. In addition, we found that SV40 TAg tumors metastasize to the lymph nodes and the liver less frequently than to the lungs (Zhang et al., unpublished data).

One limitation in testing the tumor suppressing activity of maspin in the SV40 TAg mouse model was that the transgene was dependent on the WAP promoter which was activated strongly during pregnancy and very weakly activated in the estrous cycles (Pittius et al., 1988a,b). However, once mammary cells became tumorigenic, TAg expression might become independent on the WAP promoter (Tzeng et al., 1993; Li et al., 1996). Such a change shifted the balance more towards tumorigenesis. Moreover, because endogenous maspin expression was controlled by the p53 transcription factor (Zou et al., 2000), the activation of TAg resulted in the inactivation of p53, which in turn decreased expression of endogenous maspin. These compounding effects changed the balance between positive (oncogenic) and negative (tumor suppressive) factors. In order to counteract such a potent oncogenic effect, the level of maspin expression must be increased in tumors by either systemic delivery of maspin or by placing the maspin transgene under the control of a constitutive promoter. We accomplished this goal in the following study by using a new breast tumor mouse model.

TM40D tumor implantation as a model to study breast cancer metastasis

The mammary gland is a natural site for implantation of both normal epithelial and neoplastic cells (Medina, 1996; Medina and Daniel, 1996). A frequently studied model uses serial transplantation of preneoplastic mammary outgrowth lines (Kittrell et al., 1992; Medina, 2000) that are tumorigenic and invasive. One such mammary outgrowth line, TM40D, was found to be tumorigenic and invasive (Kittrell et al., 1992; Stickeler et al., 1999). To examine maspin overexpression in mammary tumor invasion and metastasis, we established stable clones overexpressing maspin. We used the elongation factor promoter, which is constitutively active in mammalian cells, to express the maspin gene. In the first experiment, two groups of paired mice 8 weeks old were implanted in #4 mammary glands with either cells transfected with the maspin plasmid or cells transfected with control vector. Implanted control TM40D cells developed palpable tumors with 100% frequency. In contrast, only 77.8% of mice implanted with maspin transfectants developed palpable tumors (Shi et al., 2001). The tumor growth rate was also significantly decreased in maspin expressing tumors. The mean time for tumor appearance TE50 (50% of tumor endpoint) in the control group was 24 days, while palpable tumors developed slower in the maspin transfectants within 36 days ($p<0.001$).

Because some maspin transfectants lose the plasmid without antibiotic selection in vivo, we performed another experiment in which maspin was stably integrated into chromosome by retroviral insertion. When maspin stable clones were implanted into mammary gland, 84.6% of implanted sites failed to

develop tumors. The maspin clones that developed tumors also had significant reduction in growth rates compared to control tumor clones. Results from these studies showed that clones containing the integrated maspin retrovirus inhibited tumor growth more efficiently (Shi et al., 2001).

The tumor histology was also drastically different in maspin expressing clones compared to the control tumors. In general, the presence of tumor encapsulation was associated with better prognosis while the presence of excessive necrosis correlated with a more aggressive phenotype. Most sections from control TM40D tumors showed necrosis but lacked tumor encapsulation. In contrast, all maspin expressing tumors had a fibrous capsule surrounding the tumors but few had necrosis.

One of the most important features of the TM40D model is that tumors developed in the mammary gland are highly invasive and metastasize to several organs. Upon dissection of the mice, the majority of control mice had multiple tumors surrounding the intestines and some had tumors on the pleural surface, indicating that these tumors had invaded through the abdominal muscle from the #4 mammary gland. None of the maspin tumor-bearing mice had visible tumor formation on the intestine and pleural surfaces. To examine distant metastases, lung tissues from the second group of mice were collected and sectioned for microscopic analysis. Two serial sections separated by 100 μm were selected to score for micrometastatic tumor foci under high power microscopy. Our data showed that 33.3% of the control mice (four of twelve mice) developed lung metastases while none of the maspin tumor-bearing mice had any lung metastases. Overall, we observed that 75% of the control tumor mice had either invasion or distant metastases (nine of twelve mice). In addition, we observed local invasion into the muscle adjacent to the tumor and into blood vessels in the control TM40D tumor sections. However, no invasion was observed for maspin-overexpressing tumors (Shi et al., 2001).

In summary, TM40D mammary tumor cells in the syngeneic implantation model are highly invasive. Higher tumor growth rates and extensive invasion and metastases are observed in TM40D cells. Establishing a transgenic mouse model to use for overexpression studies is a lengthy process. However, one can use this model as an alternative to studying overexpression of a target gene of interest in mammary tumor progression within a short window of time. Moreover, we have recently integrated a green fluorescence protein (GFP) gene marker in the chromosome of TM40D tumor cells. Therefore, the progression of tumor metastasis can now be followed using a highly sensitive PCR analysis for detection of the marker gene in any organ of interest.

MMTV-PyV mT transgenic mice develop highly metastatic mammary tumors

Another good animal model is the MMTV polyoma middle T antigen transgenic mouse, which has been developed and characterized by Muller and Cardiff (Guy et al., 1992). These mice develop highly aggressive mammary tumors from about six weeks to three months of age, and the tumors metastasize to lung with high efficiency. The signal transduction pathway involved in tumorigenesis has also been well defined (Guy et al., 1992). The expression of transgene could activate c-src family kinases and the PI-3'-kinase signal transduction pathway (Guy et al., 1992; Webster et al., 1998; Rauh et al., 1999). Several mutations in the c-src proto-oncogene disrupted tumorigenesis induced by the PyV mT oncogene. Study of the cooperative acceleration of tumor progression between the middle T oncogene and an oncogene of interest is difficult due to the aggressiveness of PyV mT tumors. However, studying the role of tumor suppressors is feasible if the suppressor is potent enough to override the oncogenic effect of PyV middle T antigen. Furthermore, measurement of growth of the multifocal tumors that develop in the PyV mT mice is a burdensome task. In our hands, these mice develop an average of six tumors per animal by three months, and by that time the most mice must be euthanized due to the large tumor size. Surprisingly, the PyV mT tumors, although very aggressive in growth pattern, do not metastasize to any organs other than the lungs (Guy et al., 1992; Bugge et al., 1998). Histological analyses showed that many of these lung tumors were in fact well-differentiated. Data from Cardiff et al. showed that differentiation was not an appropriate marker to use for measuring the potential aggressiveness and metastasis of these tumors (see www.ccm.ucdavis.edu/tgmouse/angiogen/angiolec). Our data showed that increasing the invasiveness of PyV mT cells did not change their homing specificity. These cells metastasized only to the lung. Metastases to the lymph nodes and the liver did not occur although these metastases are common in breast cancer (Shi et al., 2002). Despite the high rate of tumor growth, MacLeord et al. have recently shown that PyV mT tumors develop following a multistep process of tumorigenesis (Maglione et al., 2001).

Conclusion

Metastasis is a complex, multistep process involving the detachment of neoplastic cells from the primary tumor, degradation of the basement membrane, tumor cell migration, intravasation and extravasation of the tumor cells and establishment in distant organs (Stetler-Stevenson, 1993). There are many factors that control the process of metastasis (Liotta et al., 1983b; Nicolson, 1991; Fidler and Ellis, 1994). Over one hundred years ago, Paget proposed a "seed and soil" theory of metastasis. The central theme of that hypothesis is that metastasis results from the specific affinity of certain tumor cells (the seed) for the milieu provided by the organs (the soil). Based on that hypothesis, certain tumor cells can metastasize only to selected organ(s). The SV40 T and TM40D models that we studied all display

high invasiveness and ability to metastasize to the lungs, a primary organ of metastasis in human breast cancer. The SV40-TAg transgenic mice develop tumors that also metastasize to the liver and lymph nodes. At the moment we have not completed the characterization of metastasis for host organs in the TM40D model. But it seems that the TM40D tumor has rather broad homing ability for host organs due to its extremely high invasiveness. A third model is the polyoma virus middle T antigen (PyV mT-high) tumor model. However, unlike the TM40D and SV40 TAg models, this tumor can metastasize only to the lung but not to other organs (Shi et al., 2002). We are interested in understanding why different tumors have such varied affinity for their host organs. Comparing the gene profiles for these different tumors will probably provide some hints on homing specificity and the mechanism of tumor metastasis. These studies are important for the basic biology as well as for the therapeutic intervention of such malignant disease in women. Animal models have been the workhorses for studying mammary tumorigenesis in the past decades, and they will become more important in our discovery of treatments for breast tumor metastasis in the future.

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