Abstract

Some clinical studies suggest that timing of surgery during specific menstrual phases may influence the chances of survival for premenopausal women with breast cancer, whereas other studies failed to find this effect. Because most breast cancer deaths are attributable to metastases, we hypothesized that aspects of the metastatic process might be sensitive to cyclic hormonal fluctuations. Our goal was to develop a mouse model to assess possible mechanisms for the effect of the menstrual cycle on metastatic ability. To separate the effects of the hormonal milieu on the host versus the cancer cells, we began by using melanoma cells. We report here that the estrous phase at the time of entry of B16F10 melanoma cells into the circulation leads to marked differences in organ-specific metastasis, suggesting that this concept merits additional study.

Introduction

The concept of scheduling breast surgery at defined menstrual phases to improve survival remains a controversial and in some cases polarizing idea (1–6). Two factors have prevented clinical application of this strategy. One is the disparity in the generally retrospective clinical data, although meta-analyses suggest that there is some validity to the concept that surgery during the luteal phase may offer some survival benefit (7–9). Second is the lack of defined mechanisms and models in which to study the phenomenon experimentally. Although the concept originally arose from studies using subcutaneously implanted murine mammary tumors (10), little experimental work has been conducted to explore potential mechanisms and no other models have been assessed.

Our objective was to develop new murine models to study the effect of the estrous cycle on aspects of the metastatic process. Both normal and tumor tissues can respond to cyclic hormonal fluctuations with changes in gene expression and many such genes have been implicated in cancer (11, 12). We have previously shown that human breast tumors resected at different phases of the menstrual cycle vary in their expression of several genes associated with malignancy (13). Surgical resection of cancer can result in shedding of tumor cells into the circulation (14–16). Cells shed during surgery thus may vary in their ability to establish metastases when tumors are resected at different times of the cycle. We hypothesized that tumor cells introduced into the circulation at different phases of the estrous cycle may vary in their ability to establish metastases and that host tissues may also vary in their ability to support metastatic growth at different phases of the cycle.

Materials and Methods

Cell Culture. B16F10 murine melanoma cells of C57BL/6 origin were purchased from the American Type Culture Collection (Manassas, VA) and grown in αMEM (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma Chemical Co., Mississauga, Ontario, Canada). The cells were maintained in a humidified incubator with 5% CO2 at 37°C and passaged at subconfluence. For injection, cells were trypsinized, washed with PBS, and resuspended at a concentration of 5 × 105 cells/ml PBS.

Estrous Cycle Phase Determination. Female C57BL/6 mice (6–7 weeks old) were purchased from Harlan (Indianapolis, IN) and cared for in accordance with the standards of the Canadian Council on Animal Care under an approved protocol of the University of Western Ontario Council on Animal Care. The animals were housed four/cage on a 12-h day/night light cycle. Vaginal sampling took place daily 3–4 h after light onset for at least one full estrous cycle before tail vein injection. Samples were obtained by vaginal lavage, the washout deposited on a glass slide, mixed with a drop of Toluidine Blue, and coverslipped. Slides were examined using light microscopy and estrous phase determined based on standard vaginal cytology (17, 18).

Experimental Metastasis Assay. Animals determined to be in either proestrus or metestrus were injected i.v. via tail vein with 100 μl of the tumor cell suspension (5 × 104 cells/mouse). Mice were autopsied 24 days later (proestrus n = 17, metestrus n = 19) and metastatic burden to lungs and extrapulmonary organs assessed by examination with a dissecting microscope. In addition, some mice were autopsied 7 days after injection (proestrus n = 12, metestrus n = 12) and examined for extrapulmonary micrometastases. Tissues were then fixed in 10% formalin, embedded in paraffin, and 4-μm sections were stained with H&E using standard procedures.

Results and Discussion

To mimic the shedding of cells at surgery, we injected cells i.v. in mice at two different phases of the estrous cycle. To separate the effects of the hormonal milieu on the host versus the cancer cells, we used B16F10 murine melanoma cells, a hormone-independent, non-breast cancer cell line. B16F10 cells were injected i.v. via tail vein; cells injected by this route will initially be taken to the lung where the vast majority will arrest, and only a very small percentage of cells will pass through the lung microcirculation, through the heart, and will be distributed systemically via the arterial circulation (19, 20).

As expected, there was a large metastatic burden in the lung at 24 days. The numbers and sizes of lung metastases did not differ for mice injected in proestrus versus metestrus (Fig. 1). Surprisingly, however, we found unexpected and dramatic differences in the organ specificity of extrapulmonary metastases when cells were injected during proestrus versus metestrus. Of the mice injected in metestrus, 31.6% (6 of 19) had prominent ovarian metastases by 24 days (Fig. 2A), whereas none of the mice injected in proestrus (0 of 17) had ovarian metastases (Fig. 2B). At 7 days after injection, ovarian micrometastases were evident in 16.7% (2 of 12) of the mice injected in metestrus, and none were detected in the mice injected in proestrus (0 of 12).

The ovarian metastases appeared to have developed within corpora lutea because micrometastases were consistently found within corpora lutea and not in any other structure or region of the ovary (Fig. 2, C and D). Both groups also had small numbers of extrapulmonary
metastases in other sites (e.g., the peritoneal cavity, abdominal mesentery, or mesenteric lymph nodes), but the incidence of nonovarian, extrapulmonary metastases between the two groups, did not differ significantly (all \( P < 0.5 \), z test; data not shown). The presence of ovarian and other extrapulmonary metastases did not correlate with the degree of metastatic burden in the lungs in either group, suggesting that the presence of ovarian (or other extrapulmonary) metastases was not a result of secondary metastasis from the lung.

This study demonstrates for the first time that the estrous phase of mice can influence the metastatic properties of cancer cells injected i.v. in experimental metastasis assays. This novel finding suggests that the fluctuating hormonal milieu of the host may differentially affect the interactions of circulating tumor cells with secondary tissues in the establishment of metastases. Our use of a hormone-independent, nonbreast cancer cell line for this study suggests that this finding is not likely attributable to a direct effect of cyclic hormones on the cancer cells but rather to a hormone-sensitive effect on the host leading to differences in some aspect of the metastatic process. These results provide an intriguing suggestion that the hormonal status of the host at the time of entry of tumor cells into the blood stream can determine whether metastases form in specific organs, even for hormone-independent tumors. These results may also be of interest to researchers using female mice to study metastasis because the estrous phase, if not controlled for, can introduce a confounding factor in the experiments.

Because cells injected via the tail vein in mice must pass through the lung as a first-pass filter where most will be arrested (19, 20), possible explanations for the difference in ovarian metastases between mice injected in metestrus versus proestrus include differential delivery of small numbers of melanoma cells to the ovaries or differential support of growth of cells in the ovary, depending on the estrous phase. Differential delivery may result from the changes in blood flow to the ovary that occurs over the estrous cycle. During metestrus, blood flow to the ovary is increased as the corpus luteum develops and by proestrus blood flow decreases as the corpus luteum regresses (21–23). Differential support of initiation of growth may result from...
the changes in tissue gene expression that occurs over the estrous cycle (11–13). Our detection of micrometastases at 7 days, in mice injected in metestrus but not proestrus, is consistent with differential delivery of cells to the ovaries at different estrous phases. However, similar (but small) numbers of cells might be delivered to the ovaries in both phases, and early events in the metastatic process could lead to micrometastatic growth only in metestrus. Because cyclic hormones can affect both vascular function and tissue gene expression, both ideas of differential delivery and differential growth warrant additional investigation.

Although the human menstrual cycle and the rodent estrous cycle differ substantially in length, proestrus corresponds to the estrogen-dominated follicular stage, and metestrus corresponds to the progesterone-dominated luteal stage (24). Although our results in a murine model cannot yet be translated directly to benefit patients, they demonstrate that the estrous phase at the time of tumor cell entry into the circulation can dramatically affect site-specific metastatic potential in a manner that is independent of hormone responsiveness of the tumor cells. Thus, the timing of surgery concept may be more broadly applicable than just to breast cancer. Additionally, this study suggests that there may be as-yet undefined subsets of patients for whom the time of surgery during the menstrual cycle may be important, whereas in other patients the timing may have no effect on their outcome. For example, the menstrual phase of surgery may be important for patients who have not had tumor cells shed from a primary tumor before surgery, as in our experimental studies, but may be unimportant in patients whose tumors have already shed appreciable numbers of cells before surgery. Current clinical difficulties in identifying such patient subsets might help to explain the mixed clinical results to date. We hope that this study will stimulate experimental research into this phenomenon and possible biological mechanisms. In turn, the resulting clarification of mechanisms may lead to appropriate translation to patients, perhaps through identification of subsets of both pre- and postmenopausal patients who would benefit from scheduling surgery at defined menstrual phases or by treatment with hormones before surgery.

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References


Estrous Cycle Influences Organ-specific Metastasis of B16F10 Melanoma Cells

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