A Rapid Imageable *In Vivo* Metastasis Assay for Circulating Tumor Cells

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Abstract. *Circulating tumor cells (CTCs) are of great importance for cancer diagnosis, prognosis and treatment. It is necessary to improve the ability to image and analyze them for their biological properties which determine their behavior in the patient. In the present study, using immunomagnetic beads, CTCs were rapidly isolated from the circulation of mice orthotopically implanted with human PC-3 prostate cancer cells stably expressing green fluorescent protein (GFP). The PC-3–GFP CTCs were then expanded in culture in parallel with the parental PC-3–GFP cell line. Both cell types were then inoculated onto the chorioallentoic membrane (CAM) of chick embryos. Eight days later, embryos were harvested and the brains were processed for frozen sections. The IV-100 intravital laser scanning microscope enabled rapid identification of fluorescent metastatic foci within the chick embryonic brain. Inoculation of embryos with PC-3–GFP CTCs resulted in a 3 to 10-fold increase in brain metastasis when compared to those with the parental PC-3–GFP cells (p<0.05 in all animals). Thus, PC-3–GFP CTCs have increased metastatic potential compared to their parental counterparts. Furthermore, the chick embryo represents a rapid, sensitive, imageable assay of metastatic potential for CTCs. The chick embryo assay has future clinical application for individualizing patient therapy based on the metastatic profile of their CTCs.*

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Circulating tumor cells (CTCs) are of great importance for cancer diagnosis, prognosis and treatment. It is necessary to improve the ability to image and analyze them for their biological properties which determine their behavior in the patient.

In a previous study from our laboratory (1), we demonstrated that CTCs isolated from PC-3 human prostate cancer expressing green fluorescent protein (GFP) or red fluorescent protein (RFP), implanted in nude mice, have higher metastatic potential than the parental PC-3 cells. However, determining the metastatic potential of CTCs in nude mice requires extensive *in vitro* culture and subsequent orthotopic implantation which takes many weeks or even months.

In another study from our laboratory using the GFP-expressing PC-3 orthotopic model and immunomagnetic beads coated with anti-epithelial cell adhesion molecule (EpCAM) and anti-anti-prostate specific membrane antigen (PSMA), GFP-expressing CTCs were isolated within 15 minutes and were readily visualized by GFP fluorescence. It was possible to immediately place the immunomagnetic bead-captured GFP-expressing PC-3 CTCs in 3-dimensional sponge cell culture where they proliferated (2).

The chick embryo model has been used to study metastasis of human cancer (3). The highly vascularized nature of the chorioallantoic membrane (CAM) allows spontaneous metastasis, enabling rapid tumor formation within several days following cancer cell implantation, in contrast to weeks or months in the mouse model. The dense capillary network within the CAM functions initially as a place for arrest, then for tumor cell extravasation and colonization (4). For these reasons, implantation of cancer cells on the CAM reliably and reproducibly results in the establishment of both primary tumors on the CAM surface, as well as generation of multiple metastatic foci.

We previously reported the development of an imageable fluorescent chick embryo model of metastatic cancer with the use of GFP expressing cancer cell lines. Lewis lung carcinoma cells, stably expressing GFP, were injected on the 12th day of incubation in the chick embryo. GFP-Lewis lung carcinoma metastases were visualized by fluorescence after seven days additional incubation in the brain, heart, and sternum of the developing chick embryo, with the most frequent site being the brain (5).

In the present study, we compared the metastatic potential of GFP-labeled PC-3 parental cells and immunomagnetic bead-isolated PC-3–GFP CTCs transplanted into chick embryos. The number of metastatic colonies established in the brain served as a quantitative measure of metastatic potential. The goal of this study was to rapidly characterize the metastatic potential of CTCs in order to move beyond their utility as simply a biologic marker (6-8).

Materials and Methods

Cell culture. The PC-3-GFP cell line has been described previously (1). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and streptamycin (Life Technologies, Inc., Carlsbad, CA, USA). Once PC-3 CTCs were isolated and immunobeads were removed, cells were stably passaged in T-75 flasks in the above medium.

Animals. Female athymic *nu/nu* nude mice were maintained in a barrier facility on high efficiency particulate air (HEPA)-filtered racks at Anticancer Inc. The animals were fed an autoclaved laboratory rodent diet (Tekland LM-485; Western Research Products, Orange, CA, USA). All surgical procedures were performed under anesthesia with an intramuscular injection of 0.02 ml solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate. Euthanasia was achieved by injecting 0.05 ml of the same solution, followed by cervical dislocation. All animal studies were conducted in accordance with principles and procedures outlined in the NIH Guide for the Care and Use of Animals under Assurance Number A3873-1.

Fluorescent orthotopic models of human prostate cancer metastasis in nude mice. For orthotopic implantation of PC-3–GFP, mice were anesthetized and positioned supinely. An opening was made right above the pubis symphysis to expose the prostate gland. The fascia surrounding the ventral portion of the prostate was carefully isolated and the two ventral lateral lobes of the gland were separated by a small incision using a pair of fine surgical scissors. Five tissue fragments of PC-3–GFP, previously grown subcutaneously, were sutured into the incision using an 8-0 nylon suture. The two parts of the separated lobes were then sutured together with the tumor fragments wrapped within. The surrounding fascia was then used to wrap this portion of the gland to consolidate the incision. The abdomen was closed using a 6-0 suture (9).

Immunomagnetic separation and enrichment of human prostate CTCs from the orthotopic mouse model. Blood (0.5-1.0 ml) was obtained by cardiac puncture. The blood was put into an EDTA tube (BD, Franklin Lakes, NJ, USA) and processed immediately. Immunomagnetic beads (AdnaTest ProstateCancer Select Kit,

AdnaGen AG, Langenhagen, Germany) were added to the tube according to the manufacturer's protocol. The bead-captured cells were then suspended in PBS or culture medium and observed under fluorescence microscopy (2).

Model of spontaneous metastasis in the chick embryo. Both PC-3–GFP parental cells and PC-3–GFP CTCs grown in culture were isolated and suspended at a density of 10^6 cells/50 μl RPMI 1640 medium. Embryos 1-3 were inoculated with PC-3–GFP cells and embryos 1-2 were inoculated with PC-3–GFP CTC cells. 5×10^5 cells/50 μl RPMI were inoculated in embryos 4-6 in the PC-3–GFP group and in embryos 3-6 in the PC-3–GFP CTC group. Approximately 70% of the embryos were expected to survive the inoculation process. The cells were introduced through a small window made in the shell above the lowered CAM on day nine of egg incubation. CAM lowering is performed by making an air pocket between the separated shell membrane and the CAM. The invasion of PC-3–GFP and PC-3–GFP CTCs was monitored by fluorescence real-time imaging with the OV-100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan) on days 1, 2, 3 post-inoculation. On day 8 post-innoculation, the CAMs were imaged with the FluorVivo Imaging System (INDEC BioSystems, Santa Clara, CA, USA) to monitor the presence of primary tumor. On day 8, brains were removed whole, placed into frozen section medium, frozen in liquid nitrogen, and then placed into a freezer at –80°C for 24 hours.

Cryosectioning. Samples were cut into 5 μm and 10 μm sections with a Leica model CM18 cryostat, and placed onto glass slides. Three representative slides were made at various depths from each chick embryo brain.

Fluorescence imaging of frozen sections. The IV-100 Intravital Laser Scanning Microscope (Olympus Corp., Tokyo, Japan) was used for fluorescence imaging of frozen-section slides. The ×20 objective was used in conjunction with an ×2 optical zoom. Gain and offset were varied during identification of fluorescent metastatic foci in order to maximize contrast with surrounding tissue and optimize data collection. Fluorescent metastatic colonies of cells in the brain were identified and counted in five separate high-power fields per sample. Three slides were examined per embryo for a total of 15 data points each and counts for each slide were then averaged.

Statistical analysis. Data were analyzed using two-way ANOVA. Comparison of PC-3–GFP parental cells and PC-3–GFP CTCs showed that the difference in brain metastasis was highly significant ($p < 0.0001$).

Results

Embryos each were inoculated with 10^6 of either PC-3–GFP cells or PC-3–GFP CTCs. In the parental PC-3–GFP group, an average of 13.8, 8.3, and 7.8 brain metastases, respectively, were found per embryo (Figure 1). In the PC-3–GFP CTC group, embryos 1 and 2 had an average of 36.2 and 33.6 brain metastases, respectively, and the third embryo in this series did not survive. This represents over a three-fold increase in metastatic lesions in the CTC group compared to the parental cells. Additional embryos were inoculated with 5×10^5 cells of either PC-3–GFP cells or PC-3–GFP CTCs. In the parental
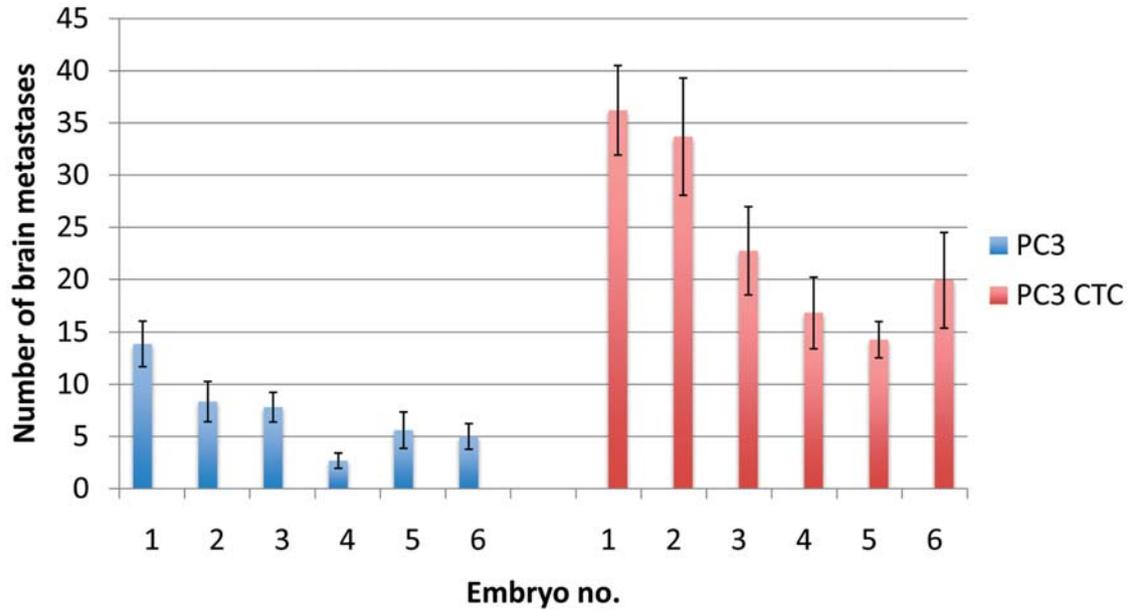
Figure 1. *Graphic representation of chick embryo brain metastasis in CAMs inoculated with PC-3–GFP parental cells vs. PC-3–GFP CTCs. Embryos 1-3 in the PC-3–GFP group and embryos 1-2 in the PC-3–GFP CTC group were inoculated with 10^6 cells each. Embryos 4-6 in the PC-3–GFP group and embryos 3-6 in the PC3–GFP CTC group were inoculated with 5×10^6 cells each. Bars represent the average number of brain metastases in each embryo (n=15 data points per embryo). The PC-3–GFP CTC group had significantly more metastases than the PC-3 parental group (p<0.0001). Error bars represent the standard error of the mean.*
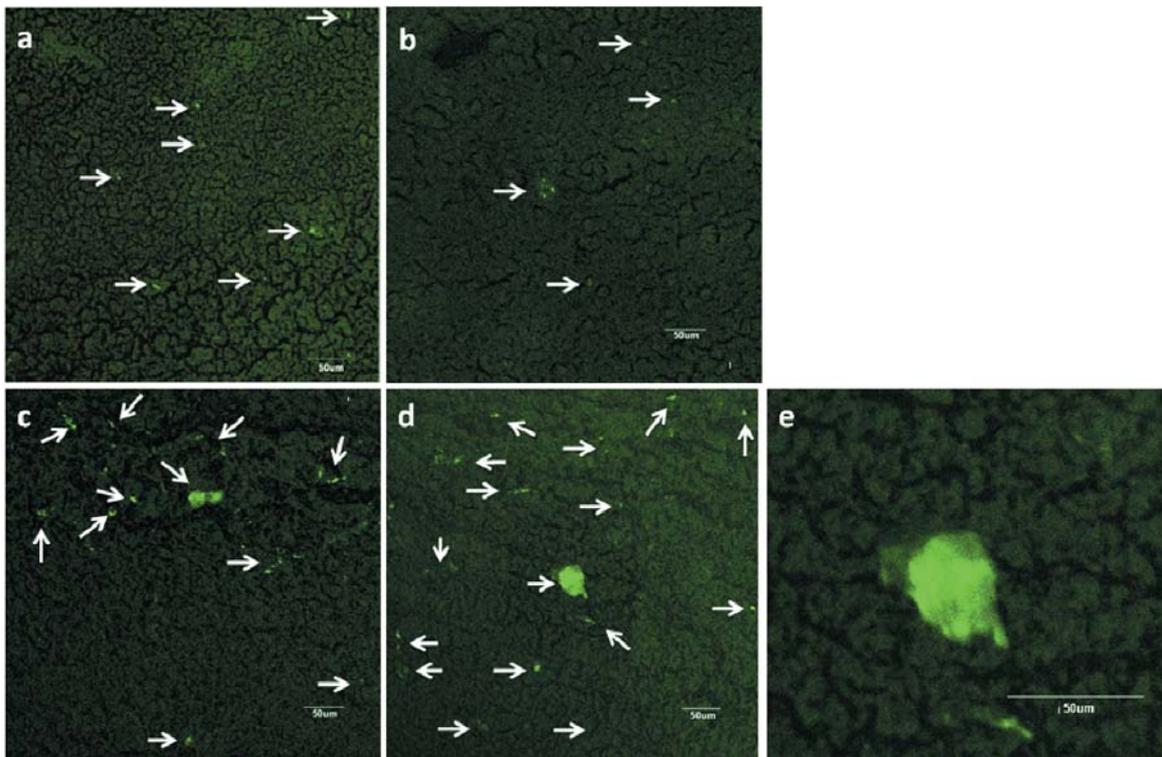

Figure 2. *Comparison of chick embryo brain metastases in CAMs inoculated with PC-3–GFP parental cells vs. PC-3–GFP CTCs. a-b: PC-3–GFP parental cell line showing sparse metastatic foci in two typical embryos. c-d: Metastases in the PC-3–GFP CTC group. All fields were counted on the Olympus IV-100 at ×20 magnification with ×2 optical zoom. e: Magnification of large PC-3-CTC metastasis at ×20 magnification with ×8 optical zoom. Arrows indicate brain metastasis.*

PC-3–GFP group, an average of 2.6, 3.6, and 5 brain metastases were found, respectively (Figure 1). In the PC-3–GFP CTC group, embryo 3 had an average of 22.7 colonies, embryo 4 had an average of 16.8 colonies, embryo 5 had an average of 14.3 colonies and embryo 6 had an average of 19.9 colonies. Although the overall number of colonies was reduced with the smaller cell inoculum, the magnitude of difference in metastasis between the CTC and parental groups remained the same.

Metastasis could be observed in all surviving embryos that were inoculated. This indicates that in the case of human prostate cancer, the chick embryo model is a reliable and reproducible assay of metastasis, even with a relatively low number of cancer cells. Within the brain tissue, the PC-3–GFP CTC group showed a greater metastatic propensity, with metastatic colonies identified in nearly all surveyed fields of brain tissue (Figure 2). In contrast, the group inoculated with parental cells had large areas of brain tissue without any evidence of metastasis (Figure 2).

Discussion

In the chick embryo, the brain serves as an ideal target organ to observe metastasis since inoculation of both the CTCs and parental cell lines resulted in micrometastasis in 100% of viable embryos. Initially, the liver was examined as another potential target organ; however, strong autofluorescence due to bile interfered with counting of metastases.

Comparison of the nature of CTCs to that of the parental cell line is important for the understanding how an aggressive tumor subpopulation is able to accomplish the complex process of metastasis. In the future, isolation of CTCs from patient blood, including those labeled ex vivo with a cancer-specific GFP adenovirus (10), and subsequent inoculation into the chicken embryo may offer a rapid assay for understanding metastatic potential on an individual basis.

The chick embryo assay for CTC metastasis, described in the present report, has important advantages for human patients. Obtaining CTCs from a patient is non-invasive and can be done as part of a longitudinal study. Studies of CTCs in the chick embryo can monitor changes in metastatic capability, drug sensitivity and viability of CTCs during the natural history of a cancer or during a treatment course since CTCs are a critical target for therapy. Since CTCs are metastatic precursors, the chick embryo assay for CTCs has the possibility to be an important tool in the improvement of therapy for metastatic cancer.

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