Abstract. We have previously demonstrated the increased metastatic potential of human prostate cancer circulating tumor cells (CTC), compared to their parental cells, in both orthotopic mouse models and the chick embryo model. In the current study, we asked whether an extracellular matrix (ECM), produced by human foreskin fibroblasts in culture, could inhibit PC-3 human prostate cancer CTC metastasis in the chick embryo model. The chorioallantoic membranes (CAM) of 18 chicken embryos were inoculated with either PC-3 human prostate cancer cells or PC-3 CTCs, both stably expressing green fluorescent protein (GFP). Embryos were divided into six groups: PC-3 parental-cell control; PC-3 plus soluble ECM; PC-3 parental cells plus semi-solid ECM; PC-3 CTC control; PC-3 CTC plus soluble ECM, and PC-3 CTC plus semi-solid ECM. Twelve hours following inoculation of the cells, a single dose of 100 μl of either soluble or semi-solid ECM was added to the appropriate group. Embryo brains were removed on day 8 post-inoculation, and were processed for cryosectioning. Imaging was performed on the cryosections using a scanning laser microscope in order to count metastatic foci. PC-3 controls had an average of 11.1 metastatic foci compared to 2.55 in the PC-3 plus soluble ECM group and 2.76 (p<0.0001) in the PC-3 plus semi-solid ECM group (p<0.0001). ECM treatment had even greater efficacy on the CTC cells, with an average of 30.9 metastatic foci in the CTC controls compared to 4.38 in the CTC plus soluble ECM group (p<0.0001) and 4.18 in the CTC plus semi-solid ECM group (p<0.0001). The results demonstrate that reduction of CTC metastatic potential is possible, in this case with an ECM produced by human foreskin fibroblasts in culture.

At the time many patients are diagnosed with cancer, circulating cancer cells (CTCs) probably exist. Therefore, the isolation and characterization of pancreatic cancer cells in the peripheral circulation could be used to design individualized therapy, as well as for prognosis (1). In our previous study, the expression levels of human telomerase reverse transcriptase (h-TERT), Keratin 20 (CK20), carcinoembryonic antigen (CEA), and C-MET were detected on CTCs of pancreatic cancer that were enriched using immune-magnetic separation (1). Using orthotopic and subcutaneous models of hormone-independent PC-3 human prostate cancer in nude mice, marked by green fluorescent protein (GFP) and red fluorescent protein (RFP), we demonstrated that viable CTCs were produced only in the orthotopic model. With fluorescence-based separation and culture of the viable CTCs, we demonstrated their increased metastatic potential compared to parental cells upon orthotopic implantation in nude mice (2).

Using the GFP-expressing PC-3 orthotopic model and immunomagnetic beads coated with anti-epithelial cell adhesion molecule (EpCAM) and anti-prostate-specific membrane antigen (PSMA), GFP-expressing CTCs were isolated within 15 minutes from nude mice 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 Gelfoam® culture, where they proliferated. The combination of GFP expression and immunomagnetic beads is a very powerful method to isolate CTCs for either analysis or biological implantation in vivo or in 3-dimensional culture (3).
The chick embryo model has been used to study metastasis of human cancer (4). The highly vascularized nature of the chorioallantoic membrane (CAM) enables rapid tumor and metastasis formation within several days following cancer cell implantation (5).

In a previous study, PC-3-GFP CTCs and parental PC-3-GFP cells were inoculated onto the CAM of chick embryos. Eight days later, embryos were harvested and the brains were processed for frozen sections. An IV-100 intravitral laser scanning microscope demonstrated a 3- to 10-fold increase in brain metastasis when compared to the parental PC-3-GFP cells (p<0.05 in all animals). The chick embryo is therefore a rapid, sensitive, imageable assay of metastatic potential for CTCs (6).

In the present study, using the chick embryo CTC metastatic assay, we demonstrate that an extracellular matrix (ECM), produced by human foreskin fibroblasts in culture, is a strong inhibitor of CTC metastasis.

**Materials and Methods**

**Cell culture.** The PC-3 human prostate cancer cell line expressing GFP used in this study has been described previously (7). Except where noted, the cells were grown to 70–80% confluence in RPMI-1640 supplemented with 10% fetal bovine serum and gentamycin (Life Technologies, Inc., Carlsbad, CA, USA), as described previously (2, 3, 8-10).

The primary fibroblast strain used in these studies was isolated from foreskin, tested for human immunodeficiency virus (HIV) I and II; hepatitis A, B and C; and human T-lymphotropic virus (HTLV) 1 and 2. It is kept as a master cell bank at passage 3 and a secondary cell bank at passage 6.

**Animals.** Male 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 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 (6).

**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 directly above the pubic 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 (7-10).

**Chick embryo metastasis assay.** PC-3–GFP and PC-3 CTCs were grown in culture, isolated and suspended at a density of 10⁶ cells/50 μl RPMI-1640 medium. The cells were introduced through a small window made in the shell above the lowered CAM on day nine of incubation. The CAM lowering was performed by making an air pocket between the separated shell membrane and the CAM. The CAMs of 18 chicken embryos were inoculated with either 10⁶ PC-3 or PC-3 CTCs (6). Twelve hours later, embryos were divided into 6 groups each containing three embryos: PC-3 parental control; PC-3 plus soluble ECM; PC-3 plus semi-solid ECM; CTC control; CTC plus soluble ECM; and CTC plus semi-solid ECM. A single dose of 100 μl of soluble or semi-solid ECM was added to the appropriate group. On day 8 post-inoculation, the CAMs were imaged with the FluorVivo Imaging System (INDEC BioSystems, Santa Clara, CA, USA) to monitor the presence of primary tumor. Brains were removed in 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 (6).

**Fluorescence imaging of frozen sections.** An IV-100 intravitral laser scanning microscope (Olympus Corp., Tokyo, Japan) was used for fluorescence imaging of frozen-section slides. A ×20 objective was used in conjunction with a ×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 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 (6).
Statistical analysis. Three slides were examined per embryo for a total of 45 data points and counts for each slide were averaged. The Student’s t-test was used to compare controls to treated groups. Data were analyzed using two-way ANOVA.

Results

We have previously demonstrated the increased metastatic potential of human prostate cancer CTCs compared to their parental counterparts using the chick embryo model (6), as well as the nude mouse orthotopic model (2). In the current study, we asked whether an ECM produced by human foreskin fibroblasts in culture could inhibit prostate CTC metastasis in the chick embryo model.

Parental PC-3 and PC-3 CTCs were inoculated onto the CAM of chick embryos. PC-3 controls had an average of 11.1 metastatic brain foci in the chick embryo compared to 2.55 in the PC-3 group treated with soluble ECM \( (p<0.0001) \) and 2.76 in the PC-3 group treated with semi-solid ECM \( (p<0.0001) \). The treatment showed even greater efficacy on the CTC cells with an average of 30.9 metastatic foci in the untreated control group compared to 4.38 in the CTC group treated with soluble ECM \( (p<0.0001) \) and 4.18 in the CTC group treated with semi-solid ECM \( (p<0.0001) \) (Figure 1).

Because all cell lines used were engineered to stably express GFP, fluorescence imaging using the IV-100 scanning laser microscope enabled rapid identification of metastatic foci in cryosections of the chick embryo brain. In the control CTC group, multiple metastatic foci were readily identifiable in all high-powered fields of cryosections of the brain surveyed (Figure 2A), and to a lesser extent in the majority of the PC-3 control (Figure 3A). In contrast for all treated groups, for both PC-3 and CTCs, large areas of each cryosection slide had to be scanned in order to identify a single metastatic focus (Figure 2B and C and Figure 3B and C).

Discussion

CTCs are potential precursors of metastasis. They are also of use in diagnosing malignancy and for prognostic purposes. Our previous study found that orthotopic tumors produced CTCs in contrast to s.c. tumors, which may explain why orthotopic tumors metastasize and s.c. tumors do not (2). This study, with the human PC-3 prostate carcinoma line, demonstrated that viable CTCs produced from the orthotopic prostate tumors have increased malignant potential compared with the parental cells in the primary orthotopic tumor. This was clearly shown in color-coded experiments where previously-selected GFP-expressing and red fluorescent protein (RFP)-expressing parental cancer cells were co-implanted in the prostate of nude mice. Resulting metastases were almost exclusively from the selected GFP-CTCs (2).
Subsequently, using the GFP-expressing PC-3 orthotopic model and immunomagnetic beads coated with anti-EpCAM and anti-PSMA, GFP-expressing CTCs were isolated within approximately 15 minutes after obtaining blood and were readily imaged 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. The combination of GFP expression and immunomagnetic beads is a very powerful method to obtain living CTCs (3).

In the chick embryo, the brain serves as an ideal target organ to observe metastasis as inoculation of both the PC-3 CTCs and parental cell lines resulted in micrometastasis in 100% of viable embryos (6).

The chick embryo assay for CTC metastasis has important potential 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 allow monitoring of changes in metastatic capability, drug sensitivity and viability. Since CTCs are metastatic precursors, the chick embryo assay for CTCs has the possibility of being an important tool to discover CTC-targeting drugs for therapy for metastatic cancer (6). The results of the present study demonstrate that reduction of CTC metastatic potential is possible, in this case with an ECM produced by human foreskin fibroblasts in culture.

In the future, isolation of CTCs from a patient’s blood, including those labeled \textit{ex vivo} with a cancer-specific GFP adenovirus (12) and subsequent inoculation into the chicken embryo can offer a rapid assay for predicting treatment response and evaluating therapeutics on an individual basis (6).
Conflict of Interest

None of the Authors have a conflict of interest in regard to this study.

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