Orthotopic Mouse Models of Tumor Metastasis Expressing Fluorescent Reporters Produce Imageable Circulating Tumor Cells

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Abstract Circulating tumor cells (CTC) are of high importance, since they are potential metastatic precursors and are readily available for prognostic analysis and treatment testing. In this review, we demonstrate the great power that green fluorescent protein (GFP) labeling and orthotopic mouse models of cancer confer to the study of CTCs for isolation and characterization, including metastatic testing in mice and the chick embryo as well as drug response testing in vitro. We also describe a facile method to label patient CTCs ex vivo using a telomerase-expressing GFP-containing adenovirus that will allow the CTC studies described in this review to be translated clinically.

Keywords CTC · Mice · Orthotopic · GFP · RFP · Isolation · Metastasis · Drug response

Introduction

Circulating tumor cells (CTC) are potential metastatic precursors and are a readily available source of cancer cells to study the properties of patient tumors as well as testing drug sensitivity of the patient's tumor. The present review describes orthotopic mouse models of cancer labeled with fluorescent proteins for the evaluation and characterization of CTCs. Providing a genetic reporter label to CTCs enables their tracking and identification even though they are often present in very low numbers. The genetic reporter label also enables their facile characterization in vitro and in vivo.

The importance of orthotopic models to generate viable CTCs is illustrated in the following example: The PC-3 human prostate carcinoma growing orthotopically in nude mice produced viable CTCs. This is in contrast to the ectopic PC-3 tumors, which did not deliver live cells into the circulation. These data emphasize the importance of the orthotopic tumor microenvironment to produce live CTCs. A mixture of PC-3 (CTCs) expressing green fluorescent protein (GFP) and parental red fluorescent protein (RFP)-expressing PC-3 were orthotopically co-implanted in nude mice. It was found that the GFP-expressing CTCs had increased metastatic propensity relative to the RFP-labeled parental cells (Fig. 1) [1].

The PC-3 CTCs were resistant to anoikis, which is apoptosis induced by cell detachment due to increased expression of the apoptosis inhibitory protein XIAP [2].

Color-coded imaging identified yellow-fluorescent CTCs that were produced after mixtures of RFP parental cells and GFP-expressing PC-3 CTC human prostate carcinoma cells were co-implanted in the nude mouse prostate and metastasized. The yellow fluorescent cells (Fig. 2) were purified from the circulation and implanted orthotopically resulting in aggressive metastatic lung cancer. In contrast, when the GFP-CTCs and RFP-parental cells were inoculated into the mouse prostate separately, none of the animals developed lung metastasis. All co-implanted animals had almost exclusively yellow fluorescent cells in the circulation and bone marrow. These results are consistent with the idea that spontaneous genetic exchange between cancer cells in vivo contributes to genomic instability and creation of highly metastatic cells [3].

The human prostate carcinoma CTCs described above overexpress both BMI1 and Ezh2 oncoproteins. The increased BMI1 and Ezh2 expression in cancer cells is associated with elevated levels of H2Aub1K119 and H3metK27 histones [4].

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Fig. 1 Fluorescence microscopy of a culture of circulating PC-3-GFP cells growing in vitro. **a** differential interference contrast image; **b** fluorescence image; **c** merge [1]

Use of GFP for Isolation and Characterization of the Metastatic Potential of CTCs

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 min and were readily visualized by GFP fluorescence (Fig. 3). It was possible to immediately place the immunomagnetic-bead-captured GFP-expressing PC-3 CTCs in 3-dimensional sponge cell culture (Gelfoam®) where they proliferated [5].

The PC-3-GFP CTCs described above were then expanded in culture in parallel with the parental PC-3-GFP cell line. Both cell types were then inoculated onto the chorioallantoic membrane (CAM) of chick embryos. Inoculation of embryos with PC-3-GFP CTCs resulted in a 3 to 10-fold increase in brain metastasis when compared to the parental PC-3-GFP cells (Fig. 4). Thus, PC-3-GFP CTCs have increased metastatic potential compared to their parental counterparts [6].

Use of GFP for Efficacy Testing of CTCs

In a subsequent experiment, CAMs of chicken embryos were inoculated with either PC-3 parental human prostate cancer cells or PC-3 CTCs, both stably expressing GFP. The embryos were treated with either a semisolid or a soluble extracellular matrix (ECM). 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 in the PC-3 plus semi-solid ECM group. 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 and 4.18 in the CTC plus semi-solid ECM group. The results

demonstrate that reduction of CTC metastatic potential is possible, in this case with an ECM produced by human foreskin fibroblasts in culture [7].

The in vitro chemosensitivity of circulating PC-3 human prostate cancer cells, isolated from nude mice orthotopically implanted with PC-3, was compared to that of the parental PC-3 cells. PC-3 parental cells and circulating PC-3 were both labeled with GFP. The CTCs exhibited a significantly increased sensitivity to both cisplatin and docetaxel when compared to PC-3 parental cells, with docetaxel having the greater efficacy. These results indicated the usefulness of the culture of CTCs from cancer patients' peripheral blood for chemosensitivity testing, for improved individualized therapy [8]. Yu et al [9] subsequently confirmed the utility of using CTCs, from patients, for drug sensitivity testing.

We then developed another animal model and new methods for CTC isolation for in vitro culture and characterization. Human lung cancer cell line H460 expressing RFP (H460-RFP), was orthotopically implanted in nude mice. CTCs were isolated by a size-based filtration method and cultured in vitro (MetaCell®). In vitro culture of CTCs used a polycarbonate membrane for the CTCs to initially attach. The cultured CTCs generally had blebbed outer membranes and were heterogeneous even though they originated from a single tumor. Abnormal mitosis resulting in three daughter cells was frequently observed. These and other features suggest the aberrancy and potential malignancy of CTCs isolated by size exclusion. The expression of RFP ensured that the CTCs originated from the orthotopic lung tumor. These readily isolatable, identifiable and cultivable CTCs can be used to characterize individual patient cancers and for screening of more effective treatment (Fig. 5) [10].

The presence of CTCs in the peripheral blood is associated with decreased survival, making the detection of CTCs clinically useful as a prognostic factor of disease outcome and/or a surrogate marker of treatment response [11].

Fig. 2 Genetic exchange between CTCs. **A–D** Fluorescence microscopy of a culture PC-3-GFP CTCs cultured in vitro, after isolation from the circulation of nude mice with orthotopic PC-3. **E–H** Fluorescence microscopy of a culture of parental PC-3-RFP cells. **I–L** Fluorescence microscopy of a representative culture of PC-3 CTCs isolated from mice co-implanted orthotopically with pre-selected GFP-expressing CTCs and non-selected RFP-expressing parental carcinoma cells. **M–P** Fluorescence microscopy of a representative culture of yellow-fluorescing PC-3 CTCs from nude mice co-implanted orthotopically with preselected

GFP-expressing viable CTCs and non-selected RFP-expressing parental PC-3 cells. **Q–T** Fluorescence microscopy of a representative culture of yellow-fluorescing PC-3 CTCs expanded in vitro from nude mice co-implanted orthotopically with preselected GFP-expressing viable PC-3 CTCs and non-selected RFP-expressing PC-3 cells. **A, E, I, M** and **Q**, red fluorescence channel; **B, F, J, N** and **R**, green fluorescence channel; **C, G, K, O** and **S**, merged fluorescence images; **D, H, L, P** and **T**, differential interference contrast (DIC) images [3]

Fig. 3 Immunomagnetic bead capture of primary tumor cells and CTC with subsequent in vivo culture. **A** Dissociated primary PC-3-GFP tumor cells captured by immunomagnetic beads. The primary tumor of a mouse orthotopically implanted with the PC-3-GFP human prostate tumor was excised and mechanically dissociated. The dissociated cancer cells were captured by immunomagnetic beads and observed by fluorescence imaging. **B–G** Gelfoam®

sponge gel cultures of circulating PC-3 CTC captured by immunomagnetic beads, growing in RPMI-1640 medium with 10 % FBS. **B** 3 days' culture. **C** 4 days' culture. **D** 4 days' culture. Note the immunomagnetic beads bound to the cell which contains two nuclei. **E** 2 days' culture. **F** 4 days' culture. **G** 4 days' culture [5]

Clinical Application of GFP for the Isolation and Characterization of CTCs

Using a telomerase-specific replication-selective adenovirus expressing GFP, a GFP-expressing attenuated adenovirus was constructed, in which the telomerase promoter regulates viral replication (OBP-401). Peripheral blood was drawn and incubated with OBP-401, resulting in labeling of CTCs with GFP [12, 13].

Shigeyasu et al. [14] have used the OBP-401 adenovirus labeling system to isolate and characterize both epithelial and mesenchymal CTCs which is in contrast to currently- used methods which depend on the EpCAM for capture. After fluorescence-activated sorting, the GFP-expressing CTCs were characterized by direct sequencing or mutation-specific PCR which detected various mutations in KRAS, BRAF, and KIT genes in epithelial and mesenchymal CTCs.

Fig. 4 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 laser scanning microscope at $\times 20$ magnification with a $\times 2$ optical zoom. **E** Magnification of large PC-3-CTC metastasis at $\times 20$ magnification with a $\times 8$ optical zoom. *Arrows* indicate brain metastasis [6]. Bar = 50 μm

Cytokeratin (CK)-positive and -negative colorectal cancer CTCs, can have complex aneuploidy [15]. This type of CTC may be highly malignant and therefore a metastasis precursor. Therefore a highly aneuploid CTC that lacks epithelial markers could be missed with current CTC capture techniques.

Shigeyasu's et al [14] and Kolostava's et al [10] development of CTC capture systems that function independently of epithelial or mesenchymal cell markers are major steps forward for assessing sensitivity to molecularly-targeted and other types of drugs, which is enabled by fluorescent protein labeling of the CTCs.

Fig. 5 H460-RFP human lung cancer CTC cultured in vitro. **(A)** Cell membrane blebs and vesicle-like structures were observed within 3 days of CTC culture. **(B)** Multinuclear CTCs were also observed [10]

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