Comparative Chemosensitivity of Circulating Human Prostate Cancer Cells and Primary Cancer Cells

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Abstract. *The 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 and circulating PC-3, both labeled with green fluorescent protein (GFP), were seeded in 96-well plates. The MTT assay was then performed at 24, 48, and 72 hours, comparing control cultures to cultures treated with cisplatin at 1, 2.5, 5 and 10 μm/ml, and docetaxel at 10, 20, 25 and 50 μm/ml at each time point. The circulating tumor cells (CTCs) exhibited a significantly increased sensitivity to both cisplatin and docetaxel when compared to PC-3 parental cells, with docetaxel having the greater efficacy. The future goal, based on these studies, is the culture of CTCs from cancer patients' peripheral blood for chemosensitivity testing, for improved individualized therapy.*

We previously reported that hormone-refractory human prostate carcinoma, growing orthotopically in nude mice, efficiently produced viable circulating tumor cells (CTCs) (1). This is in contrast to subcutaneous tumors of the same lineage, which did not produce CTCs. The CTCs had increased metastatic potential compared to parental cells when implanted in nude mice. These results enabled the systematic study of prostate cancer CTCs.

We subsequently showed that CTCs have increased resistance to anoikis, which is apoptosis induced by cell detachment (2). Using gene silencing and gene transfer techniques, we showed that increased expression of the apoptosis inhibitory protein (X-linked inhibitor of apoptosis protein or XIAP) contributed to anoikis resistance of the prostate cancer CTCs.

We also observed that both B lymphoma Mo-MLV insertion region 1 homolog (*BMI1*) and histone-lysine N-methyltransferase (*EZH2*) genes, which are part of the polycomb group, are amplified in prostate cancer CTCs (3).

Using the green fluorescent protein (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 of obtaining blood from mice and were readily visualized by GFP fluorescence (4). It was possible to immediately place the immunomagnetic-bead-captured GFP-expressing PC-3 CTCs in 3-dimensional sponge cell culture, where they proliferated.

In a subsequent study (5), PC-3 CTCs and parental PC-3 cells, both expressing GFP, were compared for metastatic potential after inoculation onto chorioallantoic membrane (CAM) of chick embryos. Laser scanning microscopy enabled rapid identification of fluorescent metastatic foci within the chick embryonic brain. PC-3-GFP CTCs had a 3- to 10-fold increase in brain metastasis when compared to the parental PC-3-GFP cells. Thus, PC-3-GFP CTCs have greater metastatic potential compared to their parental counterparts.

In the clinic, CTCs have been enumerated prior to the start of adjuvant or neoadjuvant chemotherapy, but little has been done to characterize the chemosensitivity of these CTCs which would provide important information for individualized cancer therapy. Isolation and culture of CTCs from patients' peripheral blood would allow their screening for effective drugs. The present report describes a chemosensitivity test for CTCs isolated from the orthotopic mouse models of prostate cancer described above.

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Materials and Methods

Cell culture. The PC-3 human prostate cancer cell line, expressing GFP, used in this study has been described previously (6). Except where noted, the cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum and gentamycin (Life Technologies, Inc., Carlsbad, CA, USA) to 70-80% confluence, as described previously (1, 4, 7-9).

Fluorescent orthotopic model of human prostate cancer metastasis in nude mice. A PC-3 GFP fluorescent orthotopic model of human prostate cancer was used based on surgical orthotopic implantation in the prostate of nude mice (6). Similarly to the parental PC-3-derived tumors, GFP-expressing fluorescent orthotopic tumors exhibit highly aggressive metastatic behavior, in contrast to tumors derived from the same lineage growing subcutaneously (*s.c.*). The orthotopic tumors recapitulate to a significant degree the clinical pattern of metastatic spread of advanced clinical prostate cancer (1, 6). Animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals under NIH Assurance No. A3873-01 (4).

Immunomagnetic separation and enrichment of human prostate cancer epithelial cells. Blood from nude mice with orthotopic PC-3-GFP human prostate cancer (0.5-1.0 ml) was obtained by cardiac puncture. Clotted and unclotted blood was put into EDTA tubes (BD, Franklin Lakes, NJ, USA). Erythrocytes were lysed (Buffer EL, Qiagen, Hamburg, Germany) and the CTCs were pelleted by centrifugation and were then suspended in 1 ml phosphate-buffered saline (PBS). Immunomagnetic beads (AdnaTest ProstateCancer Select Kit, AdnaGen AG, Langenhagen, Germany) were added to the tube according to the manufacturer's protocol. This kit enables the immunomagnetic enrichment of cancer cells *via* epithelial and tumor-associated antigens. After 10 min incubation at room temperature, the tube was placed in a Magnetic Particle Concentrator stand (DYNAL, Oslo, Norway), which attaches the beads to the wall of the tube. The attached beads were washed three times with 1 ml PBS. The bead-captured cells were then suspended in PBS or culture medium and observed under fluorescence microscopy (4).

CTC chemosensitivity test. PC-3-GFP, and PC-3-GFP CTCs were placed in 6 wells per column on 96-well plates at 5×10^3 cells/well. Cisplatin was administered at 1, 2.5, 5 and 10 μl/ml. Similarly, docetaxel was administered at 10, 20, 25 and 50 μl/ml. Absorbance was read by a Tecan Sunrise Microplate Reader using Magellan software (Tecan Systems, San Jose, CA, USA). Data were processed using Microsoft Excel and the percentage reduction in cell number was calculated using the untreated control cells representing 100% viability. The Student's *t*-test was performed to generate *p*-values comparing primary PC-3 cells and CTC proliferation at each time point for each drug concentration. A *p*-value of <0.05 was considered significant.

Growth curves. To ensure that the difference in response between PC-3 and CTC cell lines was not due to differences in their proliferation rates, PC-3 and PC-3 CTC cells were plated at varying cell densities. Six wells of PC-3 and of CTC cells were seeded at 10^3, 3×10^3 and 5×10^3 into 96-well plates. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis was carried out at 24, 48, and 72 h. Absorbance was read by the Tecan Sunrise Microplate Reader using the Magellan software (Tecan Systems). Data were processed using Microsoft Excel. The Student's *t*-test was performed to generate *p*-values comparing PC-3 and CTC growth curves at each time point and cell density. A *p*-value of <0.05 was considered significant.

Results

Comparative chemosensitivity of CTCs and parental PC-3 prostate cancer cells at 24 hours culture. At 24 h following the addition of cisplatin (1 μm/ml) (Figure 1A), PC-3 and CTC groups had viabilities of 99.9% and 96.0%, respectively ($p=0.19$). PC-3 and CTC treated with 2.5 μm/ml cisplatin had viabilities of 94.2% and 88.9%, respectively ($p=0.004$). PC-3 and CTC treated with 5 μm/ml cisplatin had viabilities of 64.9% and 62.3%, respectively ($p=0.59$), and those treated with 10 μm/ml cisplatin had viabilities of 29.4% and 15.6%, respectively ($p<0.001$).

The PC-3 and CTC cells treated with 10 μm/ml docetaxel had viabilities of 84.9% and 63.8%, respectively ($p<0.001$). PC-3 and CTC treated with 20 μm/ml docetaxel had viabilities of 62.4% and 55.5%, respectively ($p=0.04$). PC-3 and CTC treated with 25 μm/ml docetaxel showed viabilities of 61.8% and 56.6%, respectively ($p=0.37$). PC-3 and CTC treated with 50 μm/ml docetaxel showed viabilities of 30.9% and 14.3%, respectively ($p<0.001$).

Comparative chemosensitivity of parental PC-3 prostate cancer cells and CTC at 48 h culture. At 48 h following addition of chemotherapy (Figure 1B), cisplatin-treated (1μm/ml) PC-3 and CTC groups had viabilities of 80.9% and 77.9%, respectively ($p=0.61$). PC-3 and CTC treated with 2.5 μm/ml cisplatin had viabilities of 67.3% and 64.5%, respectively ($p=0.42$). The PC-3 and CTC cells treated with 5 μm/ml cisplatin had viabilities of 35.4% and 28.6%, respectively ($p=0.16$). PC-3 and CTC treated with 10 μm/ml cisplatin had viabilities of 2.7% and 0%, respectively ($p=0.07$).

PC-3 and CTC treated with 10 μm/ml docetaxel had viabilities of 70.7% and 80.1%, respectively, and marked the only occurrence in the present study where CTCs were less sensitive than PC-3 parental cells ($p=0.01$). PC-3 and CTC treated 20 μm/ml docetaxel had viabilities of 72.5% and 73.8%, respectively ($p=0.69$). PC-3 and CTC groups treated with 25 μm/ml docetaxel had viabilities of 68.3% and 65.5%, respectively ($p=0.26$). PC-3 and CTC treated with 50 μm/ml docetaxel had viabilities of 43.0% and 17.1%, respectively ($p<0.001$).

Comparative chemosensitivity of CTC and parental PC-3 prostate cancer cells at 72 h culture. At 72 h following addition of chemotherapy (Figure 1C), the cisplatin-treated (1 μm/ml) PC-3 and CTC had viabilities of 78.7% and 64.2%,

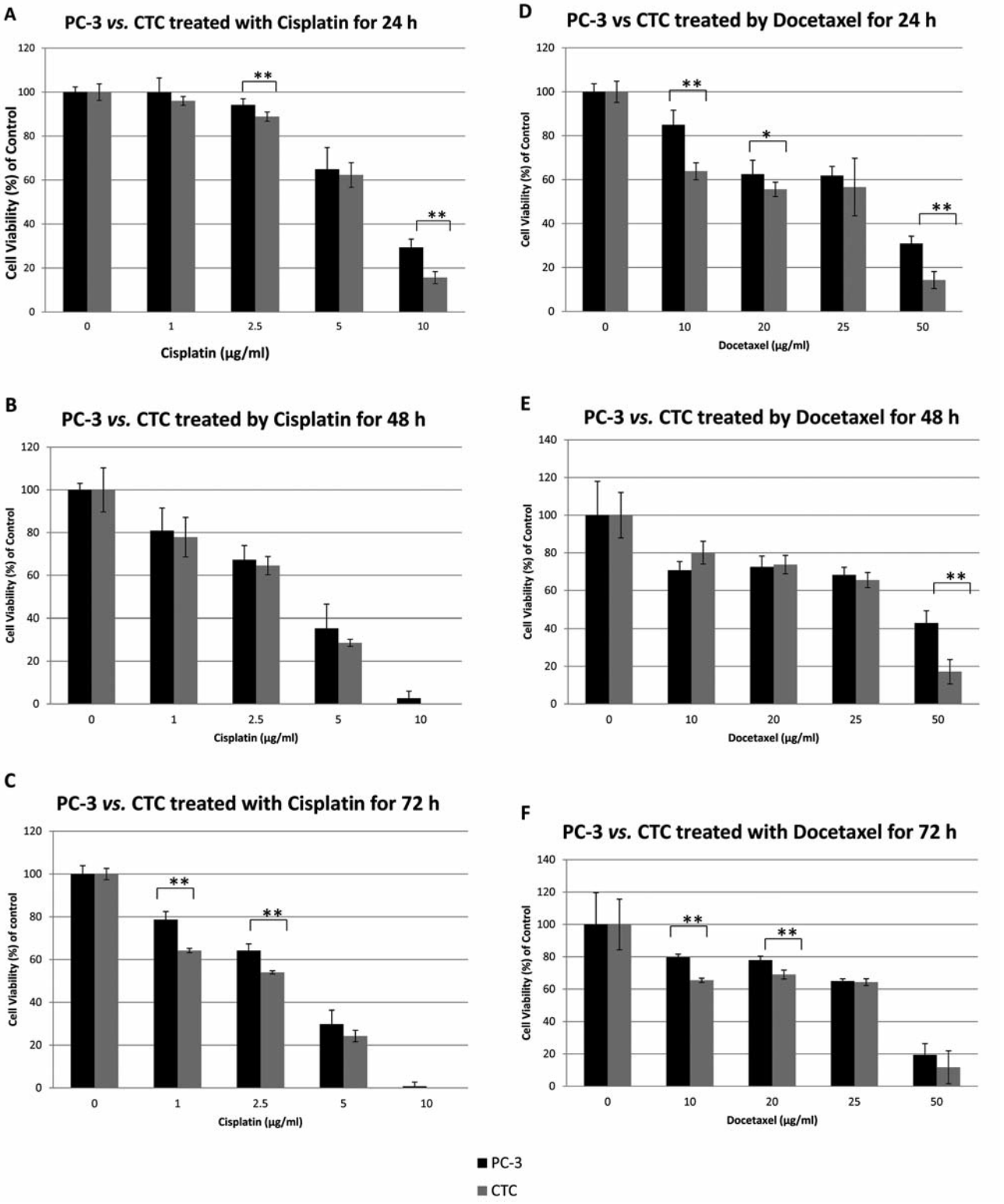

Figure 1. *A-F. Circulating tumor cells (CTC) chemosensitivity test. PC-3-green fluorescent protein (GFP) and PC-3-GFP-CTC were placed in 6 wells per column on 96-well plates at 5×10^3 cells/well. Cisplatin was administered at 1, 2.5, 5 and 10 µl/ml. Similarly, docetaxel was administered at 10, 20, 25, and 50 µl/ml. The MTT test was performed to quantify viable cells. The percentage reduction in cell number was calculated using the untreated control cells representing 100% viability. The Student's t-test was performed to generate p-values comparing PC-3 and CTC drug sensitivity at each time point for each drug concentration tested (*p<0.05 and **p<0.01).*

respectively ($p<0.001$). PC-3 and CTC treated with 2.5 μm/ml cisplatin had viabilities of 64.2% and 54.0%, respectively ($p<0.001$). PC-3 and CTC treated with 5 μm/ml cisplatin had viabilities of 29.8% and 24.3%, respectively ($p=0.085$). PC-3 and CTC treated with 10 μm/ml cisplatin had viabilities of 0.8% and 0%, respectively ($p=0.34$).

PC-3 and CTC treated with 10 μm/ml docetaxel had viabilities of 79.8% and 65.5%, respectively ($p<0.001$). PC-3 and CTC treated with 20 μm/ml docetaxel had viabilities of 77.9% and 69.0%, respectively ($p<0.001$), those treated with 25 μm/ml cisplatin had viabilities of 64.9% and 64.3%, respectively ($p=0.57$). PC-3 and CTC treated with 50 μm/ml cisplatin had viabilities of 19.3% and 11.7%, respectively ($p=0.16$).

Comparative proliferation of CTC and parental PC-3 cells. MTT assays of PC-3 and CTC cells at cell densities of 10^3, 3×10^3, and 5×10^3 cells per well were performed at 24, 48, and 72 h after cell seeding. There were no statistically-significant differences in the growth profiles of PC-3 and CTC cell lines at any time points or cell densities, with the exception when PC-3 and CTC were seeded at 3×10^3 cells/well, at 24 h ($p=0.02$).

Discussion

CTCs of PC-3 human prostate cancer had a significantly increased chemosensitivity to both cisplatin and docetaxel, with a steeper response to docetaxel. The difference in chemosensitivity between parental PC-3 and CTCs was not due to differences in proliferation rates.

In the future, isolation of a patient's CTCs will provide chemosensitivity information for individual patients without the pain, cost, impracticality, and invasiveness of repeated biopsies. CTC chemosensitivity testing will also minimize the toxicity of attempting multiple drug regimens prescribed by the current empirical method. CTCs are metastatic precursors and are arguably the most important target of chemotherapy. Thus, a CTC chemosensitivity test may revolutionize cancer treatment.

Conflict of Interest

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

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