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Observation of reversible, rapid changes in drug susceptibility of hypoxic tumor cells in a microfluidic device

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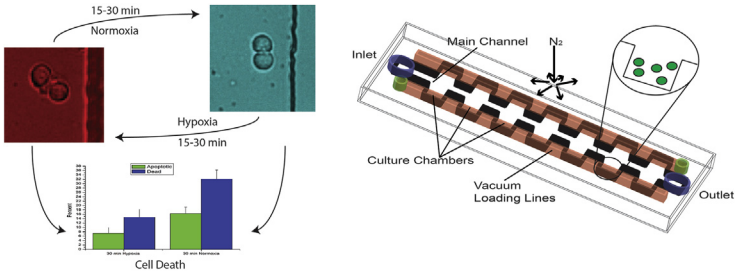
HIGHLIGHTS

- Microfluidic system switches rapidly between normoxia and hypoxia (5 min).
- Observation of rapid adaptation of PC3 cells to hypoxia and normoxia (30 min).
- Drug susceptibility in tumor cells restored after chip switched to normoxia for 30 min.

GRAPHICAL ABSTRACT



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ABSTRACT

Hypoxia is a major stimulus for increased drug resistance and for survival of tumor cells. Work from our group and others has shown that hypoxia increases resistance to anti-cancer compounds, radiation, and other damage-pathway cytotoxic agents. In this work we utilize a microfluidic culture system capable of rapid switching of local oxygen concentrations to determine changes in drug resistance in prostate cancer cells. We observed rapid adaptation to hypoxia, with drug resistance to 2 μM staurosporine established within 30 min of hypoxia. Annexin-V/Sytox Green apoptosis assays over 9 h showed 78.0% viability, compared to 84.5% viability in control cells (normoxic cells with no staurosporine). Normoxic cells exposed to the same staurosporine concentration had a viability of 48.6% after 9 h. Hypoxia adaptation was rapid and reversible, with Hypoxic cells treated with 20% oxygen for 30 min responding to staurosporine with 51.6% viability after drug treatment for 9 h. Induction of apoptosis through the receptor-mediated pathway, which bypasses anti-apoptosis mechanisms induced by hypoxia, resulted in 39.4 ± 7% cell viability. The rapid reversibility indicates co-treatment of oxygen with anti-cancer compounds may be a potential therapeutic target.

1. Introduction

Prostate cancer is the most common cancer among men, especially in developed countries [1–3]. Based on autopsy results, 30% of men 50 and older and 70% of men 80 and older have incidental prostate cancer [1]. The main risk factors for prostate cancer are age, race, and family history [3]. Prostate cancer can in some cases be treated by using hormone treatments, but in hormone resistant prostate cancer chemotherapy is typically used [4]. Resistance to chemotherapy drugs can allow for prostate cancer cells to metastasize and increases mortality rates [5]. The primary death mechanism triggered by these chemotherapy compounds is apoptosis [6–8]. However, treatment of solid tumors has proven to be difficult, in part due to adaptations in the tumor cells, resulting in

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changed regulation of pro- and anti-apoptosis factors [9].

Most chemotherapy agents that trigger apoptosis do so via the intrinsic—or cell damage—pathway [10]. This pathway can be blocked at the mitochondria by Bcl-2 and Bcl-xL regulatory proteins, which are expressed at different levels under normoxic and hypoxic conditions and are regulated in part by Hypoxia Inducible Factor 1α [11]. This effect is seen in solid tumor growths, as inner regions lack sufficient blood flow [12–16].

Hypoxia Inducible Factor (HIF)-1 is a transcription factor that is continuously present in cells during normoxic conditions. The HIF-1 heterodimer is composed of 4 subunits, 3 alpha subunit (HIF-1α, HIF-2α, HIF-3α) and 1 beta subunit (HIF-1β) [17,18]. HIF-1 is continuously degraded under normoxia by the HIF hydroxylase family of enzymes [19]. These hydroxylases require molecular oxygen, among other compounds to maintain their activity [20]. As the oxygen concentration in the cell decreases, HIF-1 accumulates and begins regulating cell survival mechanisms, among other phenotypic changes [9,21,22]. Hypoxia can either prevent or initiate apoptosis by changing the expression of over 70 genes regulated by HIF-1 [23]. The expression of anti-apoptosis proteins Bcl-2 and Bcl-xL is increased under hypoxic conditions. These proteins block the ability of Bax and Bak to start the apoptosis cascade in the mitochondria [24]. Understanding the temporal dynamics of hypoxia can lead to new therapeutic strategies for tumors, but are difficult to conduct *in vivo* due to the highly variable nature of solid tumors and the inability to conduct real-time assays.

Microfluidic systems are an ideal platform to study hypoxia effects in tumor cells [25,26]. In addition to real-time control of oxygen concentrations, the ability to directly observe cell response allows many fundamental studies to be conducted [27–30]. Using poly (dimethyl siloxane) (PDMS) as the fabrication material allows for rapid switching of oxygen concentrations as well as oxygen gradients in some cases [28]. In this work, we use a microfluidic tumor culture system capable of rapid oxygen switching to study the adaptation of cancer cells to hypoxic and normoxic environments. We observed that tumor cells adapted to hypoxia within 30 min, rendering them resistant to an anti-cancer compound. We also demonstrate that the process is reversible, and drug resistance was reduced in the same timeframe when oxygen was delivered to the cells. These findings not only demonstrate the ability to study reversible cell changes in hypoxia, but also indicate that relatively brief exposures of oxygen may result in enhanced cell death in tumor cells.

2. Experimental

2.1. Materials and reagents

PDMS prepolymer and curing agent were purchased from Ellesworth Adhesives (Dow Sylgard 184). Microscope glass slides ($76.2 \times 25.4 \times 1$ mm^3) were purchased from VWR international. RPMI 1640 medium with 2.05 mM L-glutamine and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT). Trypsin EDTA was purchased from Mediatech, Inc. (Herndon, VA). Phosphate buffered saline (PBS, pH 7.4) was purchased from Gibco Invitrogen (Grand Island, NY). Rhodamine-110 Chloride was obtained from Sigma Aldrich (St. Louis, MO). Silicon wafers were purchased from University Wafer. SU-8 and SU-8 developer were purchased from MicroChem Corp. (Westborough, MA). Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride (Ru(ddp) was purchased from Sigma Aldrich (St. Louis, MO). Annexin V Alexa Fluor 647 conjugate and Sytox Green were obtained from Invitrogen (Waltham, MA).

2.2. Microfluidic device fabrication

Microfluidic devices were fabricated using standard multilayer soft lithography procedures. The device (Fig. 1) is a two layer system with culture chambers in the bottom layer and vacuum lines for cell loading in the top layer. Master molds were made by spin-coating SU-8 on a silicon wafer to the desired channel height of 40 μm. The PDMS prepolymer and curing agent (Dow Sylgard 184) were mixed together in a 5:1 ratio for the top vacuum layer. The PDMS mixture was degassed in a vacuum and then poured onto the wafer mold and baked at 95 °C for 1 h. The bottom layer was a 25:1 ratio of PDMS prepolymer to curing agent spin coated at 2000 rpm for 30 s. After baking the bottom layer for 30 min at 70 °C the two layers were aligned and baked at 120 °C for 2 h to seal the layers together. Holes for the fluid and vacuum inlets and outlets were punched and the PDMS layers were bonded to glass using oxygen plasma to complete the device.

2.3. Cell culture and loading

Human prostate cancer cells (PC3, ATCC #CRL-1435), which originate from a bone metastasis of a 62 year old patient with epithelial morphology, and Ramos B lymphocytes (ATCC #CRL-1596), which originate from B-lymphocytes of a 3 year old patient with lymphoblast morphology, were cultured in the incubator in culture flasks at 37 °C and 5% CO_2. On the day of the experiment PC3 cells were removed from the flask using trypsin. After washing the cells with PBS the cells were re-suspended in medium (RPMI + 10% FBS + 20 mL Antibiotic). For Ramos cell studies, cells were extracted from the flask and centrifuged to the necessary concentration. Cells were loaded into the culture chambers of the microfluidic device by connecting the vacuum lines to the vacuum pump until the culture chambers were full of cell suspension. Once all of the culture chambers were filled, medium was flowed through the main channel at 0.1 mL/h. Previous work has shown that at this flow rate apoptosis in control samples were comparable to a petri dish, at 168 h of culture time, the cells had a viability of 96% [27]. Fig. 2 shows prostate cancer cells after 8 h of exposure to staurosporine in brightfield (white light) and fluorescence images of Annexin V Alexa Fluor 647 and Sytox Green.

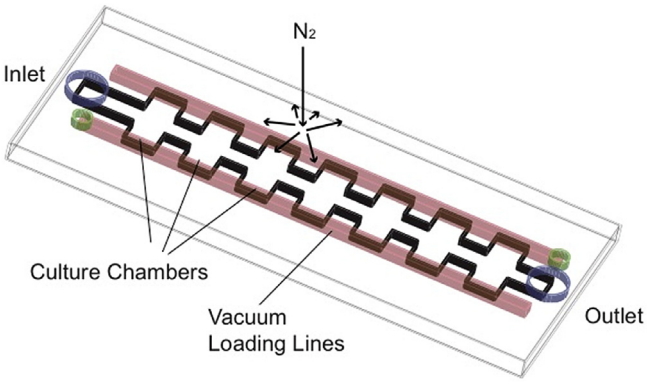

Fig. 1. Schematic of the microfluidic device used for culturing cells in this experiment. Culture chambers are loaded with cells using vacuum loading lines (red). Once loaded, cells remain in the culture chamber and are cultured under a low-shear environment. Constant medium flow through the main channel supplies nutrients to and removes waste from the culture chambers. Only a portion of the 256 culture chambers are shown for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)