Targeting Quiescent Tumor Cells via Oxygen and IGF-I Supplementation

Alastair H. Kyle, Jennifer H.E. Baker, and Andrew I. Minchinton

Abstract

Conventional chemotherapy targets proliferating cancer cells, but most cells in solid tumors are not in a proliferative state. Thus, strategies to enable conventional chemotherapy to target noncycling cells may greatly increase tumor responsiveness. In this study, we used a 3-dimensional tissue culture system to assay diffusible factors that can limit proliferation in the context of the tumor microenvironment, with the goal of identifying targets to heighten proliferative capacity in this setting. We found that supraphysiologic levels of insulin or insulin-like growth factor I (IGF-I) in combination with oxygen supplementation were sufficient to initiate proliferation of quiescence cells in this system. At maximal induction with IGF-I, net tissue proliferation increased 3- to 4-fold in the system such that chemotherapy could trigger a 3- to 6-fold increase in cytotoxicity, compared with control conditions. These effects were confirmed in vivo in colon cancer xenograft models with demonstrations that IGF-I receptor stimulation was sufficient to generate a 45% increase in tumor cell proliferation, along with a 25% to 50% increase in chemotherapy-induced tumor growth delay. Although oxygen was a dominant factor limiting in vitro tumor cell proliferation, we found that oxygen supplementation via pure oxygen breathing at 1 or 2 atmospheres pressure (mimicking hyperbaric therapy) did not decrease hypoxia in the tumor xenograft mouse model and was insufficient to increase tumor proliferation. Thus, our findings pointed to IGF-I receptor stimulation as a rational strategy to successfully increase tumor responsiveness to cytotoxic chemotherapy.

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Introduction

The microenvironment of solid tumors has long been identified as a source of resistance to chemotherapy and radiotherapy. Sustained expansion of the tumor cell population and aberrant neovascularization leads to the creation of an environment that differs from normal tissues. Deregulated proliferation and survival of tumor cells result in increased separation of blood vessels (1, 2) and unstable perfusion (3–6), in turn leading to poor efficiency in the distribution and removal of molecules supplied by the blood (7–11). For example, tumor cells can be located up to 15 to 20 cells away from the nearest blood vessel (more in some cases), whereas in most normal tissues cells are within just a few cell layers of a vessel (12). The diffusion gradient of molecules supplied by the blood creates subpopulations of cells within the tumor that will differ in proliferation status and response to chemotherapy. This phenomenon is well illustrated in corded HCT116 xenografts, when gradients in oxygenation and proliferation are mapped in relation to tumor vasculature (13). As shown in Fig. 1A, S-phase cells in HCT116 xenografts can be observed nearer to blood vessels, whereas quiescent cells predominate in areas further from vessels. Despite uncontrolled proliferation being a hallmark of cancer, human tumors often exhibit proliferative indices as low as 5% to 10% (14). Even micrometastases of a quarter of a millimeter in size will possess regions of hypoxia and nutrient deprivation leading to tumor cell quiescence (10, 15). Previous studies examining the microregional activity of paclitaxel and gemcitabine tissue mapping found that quiescent cells located far from blood vessels recovered more readily following treatment (13, 16).

Although numerous factors have been identified from in vitro studies that modify tumor cell proliferation, their relative importance in the context of the tumor microenvironment is still poorly understood. Limitations in the supply of oxygen, signaling molecules, and key nutrients such as glutamine as well as tumor acidification have all been implicated as causes of tumor cell quiescence (17–19). Despite oxygen being a critical factor in the regulation of proliferation and survival, it is unclear whether other factors may initiate quiescence in solid tumors prior to oxygen becoming limiting (15, 20, 21). To date stimulating tumor cell proliferation for therapeutic gain using strategies that focus on single factors has largely been unsuccessful and it might be that identifying more than one factor will be required (22–24). Tumor-derived quiescence is likely due to the interrelation of many diffusible factors, an issue that is compounded by the difficulty of independently...
screen were evaluated both in terms of their ability to stimulate proliferation and to resensitize quiescent cells to chemotherapy. Because any strategy that increased tumor cell proliferation between treatments would be more likely to fail, tumors were transiently stimulated prior to chemotherapy. We hoped that this approach would favorably alter the balance between tumor debulking by chemotherapy and regrowth between treatments. In theory, such a strategy would also maximize an antiproliferative therapeutic ratio of drug, as normal tissues are likely less responsive to stimulation because they possess intact regulatory pathways controlling cell growth and, due to their efficient vasculature, they will already have access to the diffusible molecules from which tumor cells have become deprived.

Materials and Methods

Monolayer and 3D tissue disk culture

HCT116 and HT29 cells were obtained from the American Type Culture Collection in 2001, and cell type was confirmed for each experiment via 3D tissue morphology and architecture as seen in histologic sections. Mycoplasma status was tested on a monthly basis using Hoechst 33342 labeling of DNA. Cells were cultured in MEM (HyClone) with 10% FBS (Gibco/BRL) under 5% O2 and 5% CO2. Three-dimensional tissue disks were grown by seeding 1 × 10^6 cells into collagen-coated tissue culture inserts (CM 12 mm, pore size 0.4 μm; Millipore) as previously described (26). Inserts were then incuated for 16 hours to allow cells to attach to the porous membrane prior to being submerged in media and transferred to stirred growth vessels (Fig. 1D).

Closed-off 3D tissue disk assay

Upon reaching approximately 125 μm in thickness HCT116 3D tissue disks were transferred from growth vessels (Fig. 1D) to penetration vessels (Fig. 1E) and immersed in stirred MEM with 10% FBS. The growth media was then supplemented with additional factors and the disks incubated for 24 hours. Supplemented factors included 22 mmol/L D-glucose (Sigma-Aldrich), 0.6 mmol/L L-arginine, 0.1 mmol/L uridine and inoline (Sigma-Aldrich), 1% ITS cocktail (10 μg/mL insulin, 5.5 μg/mL transferrin and 0.0067 μg/mL selenium; Gibco #41400) or 10 μg/mL insulin alone (insulin from bovine pancreas; Sigma-Aldrich #0516). Two hours prior to completion of 100 μmol/L bromodeoxyuridin (BrdUrd; Sigma-Aldrich) was added to the reservoir to label S-phase. Tissue disks were frozen and embedded in O.C.T. compound (Tissue Tek) in preparation for cryosectioning.

Open 3D tissue disk assay

HCT116 or HT29 tissue disks 200 to 300 μm in thickness were maintained in growth vessels (Fig. 1D) immersed in stirred MEM with 10% FBS. Media was then supplemented with additional oxygen, glucose, and insulin or in later experiments LR3-IGF-I (Long R Insulin-like growth factor I, #855803C, SAFC Global). Two hours prior to completion of each supplementation period 100 μmol/L BrdUrd and 50
μmol/L pimonidazole (Hypoxprobe-1 Kit; Chemicon International Inc.) were added to the reservoir to label S-phase and hypoxic cells. Tissue disks were frozen and embedded in O.C.T. compound in preparation for cryosectioning. For cell survival experiments, the 3D tissue disks were supplemented with 100 ng/mL LR3-IGF-1 under normal or high oxygen conditions (5 or 95% O₂) for 12 hours prior to a 6-hour cytotoxic chemotherapy exposure. Following treatment, tissue disks were rinsed and returned to fresh media and incubated for an additional 24 hours prior to dissociation using 0.25% trypsin EDTA (Sigma-Aldrich) for 15 minutes followed by 5-minute DNase treatment (1.5 mg/mL deoxyribonuclease I from bovine pancreas; Sigma-Aldrich). Cells were then counted and plated for colony-forming assay.

**Mice and tumors**

Female NODCB17-Prkdcscid/J mice were bred and maintained in our institutional animal facility in accordance with the Canadian Council on Animal Care guidelines. Experiments are approved by the Animal Care Committee of the University of British Columbia. Mice were allowed free access to standard laboratory rodent food and water and were used between 8 and 14 weeks of age, typically weighing between 20 and 30 g. HCT116 cells (8 × 10⁶ cells in 50 µL) or HT29 cells (5 × 10⁶ cells in 50 µL) were implanted subcutaneously into the sacral region and tumors grown to 150 mm³ as calculated from caliper measurement of 3 orthogonal diameters (a, b, and c) using the formula volume = π/6abc.

**Tumor mapping experiments**

Mice were administered with 1 mg/kg LR3-IGF-1 by s.c. injection at 0, 4, 8, and 12 hours alone or in combination with pure oxygen breathing under 1 or 2 atmospheres pressure. Hyperbaric work was carried out using a pressurized acrylic chamber (27). At the 12-hour time point mice were administered 1,000 mg/kg BrdUrd (Sigma-Aldrich) and 60 mg/kg pimonidazole by intraperitoneal injection and euthanized 2 hours later. Five minutes prior to carbon dioxide euthanasia and tumor harvest mice were administered 35 µL of 0.6 mg/mL DiOC₇(3) (Sigma-Aldrich) in 25% dimethyl sulfoxide to demarcate perfused vessels. Tumors were then excised, weighed, and immediately frozen.

**Tumor growth delay experiments**

Mice were administered 1 mg/kg LR3-IGF-1 by subcutaneous injection at times 0, 4, 8, and 12 hours prior to cytotoxic chemotherapy. Paclitaxel at 30 mg/kg or 120 mg/kg gemcitabine was then administered intraperitoneally. Both the LR3-IGF-1 and chemotherapy regimens were repeated once weekly for 3 weeks, except paclitaxel in HCT116 which was administered for 2 weeks only. Tumor volumes were measured 3 times per week.

**Immunohistochemistry: DiOC₇(3), CD31, pimonidazole, and BrdUrd**

Prior to immunostaining, slides were imaged for DiOC₇(3) tissue fluorescence to visualize blood flow. Cryosections were then fixed in a 1:1 mixture of acetone–methanol for 10 minutes at room temperature. Vasculature was stained using a 1:400 dilution of anti-PECAM/CD31 (BD Pharmingen clone MEC13.3) and 1:200 fluorescent Alexa 546 anti-rat secondary (Invitrogen). Hypoxia was detected via bound pimonidazole adducts using a 1:400 polyclonal rabbit–anti-pimonidazole (Hypoxprobe-1 Kit; Chemicon International Inc.) and a 1:200 Alexa 488 anti-rabbit secondary. Slides were imaged for fluorescence and then transferred to distilled water for 10 minutes and then treated with 2 mol/L HCl at room temperature for 1 hour, followed by neutralization for 5 minutes in 0.1 mol/L sodium borate. Slides were then washed in distilled water and transferred to a PBS bath, and BrdUrd incorporated into DNA was detected using a 1:500 dilution of monoclonal mouse anti-BrdUrd (clone BU33) followed by 1:200 dilution of anti-mouse peroxidase conjugate antibody (Sigma-Aldrich) and 1:10 dilution of metal enhanced 3,3′-diaminobenzidine substrate (Pierce). Slides were counterstained with hematoxylin, dehydrated and mounted using Permount (Fisher Scientific). For 3D tissue disks, the CD31 staining was skipped and a fluorescent secondary, anti-mouse Alexa 546 was used to detect the BrdUrd primary. Tissue disks were stained with Hoechst 33342 to label cell nuclei.

**Image acquisition**

The imaging system consisted of a robotic fluorescence microscope (Zeiss Imager Z1), a cooled, monochrome CCD camera (Retiga 4000R; Q Imaging), a motorized slide loader and x-y stage (Ludl Electronic Products) and customized ImageJ software (public domain program developed at NIH running on a Macintosh computer (Apple). The robotic system allowed for tiling of adjacent microscopic fields of view of entire tumor cryosections up to 1 to 2 cm² captured at a resolution of 0.75 µm/pixel.

**Image analysis of tumor xenografts**

Using ImageJ and user-supplied algorithms, images of DiOC₇(3), CD31, BrdUrd, and pimonidazole staining from each tumor section were overlaid and areas of necrosis and staining artifacts manually removed. CD31-positive regions were identified by selecting all pixels 10 SD above the tissue background levels. CD31-positive regions that were less than 10 mm² in size were considered artifacts and automatically removed from the analysis. BrdUrd-positive staining was identified by selecting pixels that were 5 SD above tissue background levels. Measuring the distance from each point in the tissue to the nearest CD31-positive object and noting whether it was BrdUrd positive or negative was used to determine the relation between proliferation and distance to the nearest detected blood vessel. The data were tabulated so as to determine the fraction of BrdUrd-positive pixels of the total number of pixels found at each distance to a blood vessel. Pimonidazole staining was assessed via similar methods, using average signal intensity rather than the fraction of pixels above threshold.

**Image analysis of 3D tissue disks**

Using ImageJ and user-supplied algorithms, images of BrdUrd, pimonidazole, and Hoechst staining from each tissue section were overlaid and staining artifacts manually removed.
Tissue edges were traced manually and then BrdUrd and pimonidazole were mapped in relation to distance from tissue edges. Three tissue sections 5 to 7 mm in length were taken from each disk.

**Statistical analysis**

One-way ANOVA tests were done using Prism software (GraphPad). Significance of differences between multiple groups was compared using a Bonferroni posttest analysis.

**Results**

**Activity-based screen using HCT116 3D tissue disks identifies that supraphysiologic insulin supplementation can reverse diffusion-limited proliferation**

The ability of a selection of media supplements to sustain proliferation on the temporarily closed-off side of 3D tissue disks was assayed over a 24-hour period. Figure 1F shows analysis of BrdUrd labeling in tissue located against the clamped side of the disk, 75 to 150 µm in from the media side. Prior to being closed off, the clamped side had access to media and exhibited equal proliferation to the open side. Of the panel of supplements tested, only the 1% ITS cocktail or 10 µg/mL insulin alone was found to maintain active S-phase status in the tissue on the closed side. Ten µg/mL insulin is approximately 100 times greater than physiologic blood concentrations, and at this level, it also acts to efficiently stimulate the IGF-I receptor and downstream AKT/mTOR pathway leading to proliferation (28–31). Follow-up studies revealed similar activity after 100 ng/mL LR3-IGF-I stimulation. FBS, glucose, glutamine, and DNA precursor supplements, uridine and inosine, were not able to sustain proliferation following the 24-hour period of closure.

**Supraphysiologic insulin in combination with oxygen supplementation yields greatest increase in proliferation in HCT116 3D tissue disks**

A second screen was carried out to determine the effect of supplements on cells in the normally quiescent zone of thicker 3D tissue disks. HCT116 disks were grown to 200 to 300 µm and then, without closing off the disks, normal growth conditions were supplemented with insulin, glucose, amino acids, and oxygen (5%, 20%, and 95% O2) for a 24-hour period. Immunostaining results for S-phase and hypoxic profiles within tissue cryosections are shown in Fig. 2 (also see Supplementary Data 1 for a comparison with IGF-I stimulation). Figure 3 summarizes analysis of the interrelationship of insulin, oxygen, glucose, and proliferation within the disks. The maximum depth of proliferation within the disks was seen to increase with increasing oxygen (Fig. 3A–C), and stimulation following insulin was more effective at higher oxygen levels (Fig. 3D–F). Results indicated that maximal proliferation occurred when both oxygen and insulin were supplemented. Under combined insulin and oxygen supplementation, the high level of proliferation of...
Transplantation of Quiescent Tumor Cells

Figure 3. Analysis of the effect of oxygen and insulin supplementation on proliferation and hypoxia in 3D tissue disks. A–F, average (avg.) BrdUrd and pimonidazole staining as a function of depth into tissue from the bottom of a disk as depicted in Fig. 2. Light gray lines show results for the individual tissue sections and black (BrdUrd) and gray (pimonidazole) lines show group averages. Total tissue area analyzed from each cryosection was approximately 10 times the area shown in the images from Fig. 2. G, average BrdUrd staining intensity over first 150 µm from the bottom edge and, H, average disk thickness. Bars show average ± SD, (n = 6–9), *P < 0.01. arb., arbitrary; ctrl, control gluc., glucose.

Figure 4. Time course of stimulation following oxygen and LR3-IGF-I supplementation in 3D tissue disks. Comparison of 5% versus 95% oxygen (LR3-IGF-I) in (A) HCT116 or (B) HT29 3D tissue disks. Peak induction occurs 12 to 24 hours following stimulation at which point quiescent areas 100 to 150 µm from tissue surface reached proliferation levels seen on tissue edge. Data show the fraction by area of each tissue section that stained positive for the S-phase marker BrdUrd (error bars show SD, n = 6). *, P < 0.01.

100 to 150 µm into the tissue ruled out the supply of other diffusible molecules as being dominant factors limiting proliferation within the tissue. Oxygen and insulin supplementation alone were enough to sustain proliferation within the tissue near levels seen on the tissue edge. Fig. 3G and 3H compare average proliferation and disk thickness following the 24-hour supplementation period. In the disks under the approximately physiologic level of 5% oxygen, glucose supplementation did not increase proliferation (Fig. 3G) but did increase the maximum depth that cells were able to survive within the tissue prior to becoming necrotic (Fig. 3H).

Time course of induction of proliferation following LR3-IGF-I and oxygen supplementation

In preparation for translation of findings to tumor xenograft-based studies, work was carried out to look at the time course of induction of proliferation in quiescent tissue using LR3-IGF-I, a synthetic analogue of IGF-I which is not bound by IGF-binding proteins in the blood and remains in an active form (32, 33). Figure 4 shows the time course of stimulation in oxygenated regions near the edge of tissue, 0- to 50-µm versus 50- to 100-µm intermediate zones and more hypoxic areas 100 to 150 µm into the tissue. As with previous work using insulin, direct stimulation of the IGF-I receptor using LR3-IGF-I in combination with oxygen supplementation was able to initiate proliferation in normally quiescent areas. Maximal induction occurred 16 to 24 hours after initiation, at which point overall tissue proliferation increased 3-±0.4-fold (HCT116) and 4-±1-fold (HT29), as measured using an S-phase endpoint (BrdUrd incorporation). Under 5% oxygen, a transient increase in proliferation occurred predominantly in regions 50 to 100 µm from the edge of the tissue but not in more hypoxic areas further inwards. This transient stimulation 8 to 16 hours after media supplementation explains the increase in disk thickness seen in Fig. 3H, despite which proliferation levels seemed normal at 24 hours, as shown in Fig. 3G.
LR3-IGF-I and oxygen supplementation in 3D tissue-disks sensitizes tissue to chemotherapy

HCT116 and HT29 3D tissue disks were stimulated with LR3-IGF-I for a 12-hour period under 5% or 95% O₂ followed by a 6-hour chemotherapy treatment period. Figure 5A shows the result for overall surviving fraction of cells from HT29 disks treated under normal versus oxygen and LR3-IGF-I supplemented conditions. Surviving fraction (SF) in chemotherapy-treated disks was seen to decrease by approximately 6-fold under combined stimulation compared with disks under normal conditions (SF 0.71 ± 0.08 reduced to 0.10 ± 0.03). Cell kill was greater when chemotherapy was given 12 hours after initiation of stimulation compared with when given simultaneously. Similar results were seen for treated HCT116 disks (SF 0.28 ± 0.1 reduced to 0.046 ± 0.007). The effect of LR3-IGF-I prestimulation on cell kill for a panel of chemotherapy agents in disks under 5% O₂ is shown in Fig. 5B, in which surviving fraction is seen to be decreased by 3- to 6-fold.

LR3-IGF-I supplementation increases response to chemotherapy in tumor xenografts

The combination of IGF-I receptor stimulation with paclitaxel or gemcitabine treatment was assayed in HCT116 and HT29 tumor xenografts. Tumor growth delay following 30 mg/kg paclitaxel (q7 × 2) in HCT116 xenografts with and without a 12-hour LR3-IGF-I stimulation is shown in Fig. 7A. Significant increases in the delays in time to reach 3-fold increase in volume were seen following LR3-IGF-I prestimulation for paclitaxel (48% increase in HCT116) and for gemcitabine (25% increase in HCT116 and 40% in HT29) as compared with unstimulated mice (Fig. 7B). No significant change in mouse weight was seen between normal mice and stimulated mice. On its own, LR3-IGF-I stimulation was not seen to alter tumor growth rates, consistent with the transient nature of the stimulation period of once per week for a 12-hour period (data not shown).

Discussion

This study shows that IGF-I receptor stimulation could be successfully used to increase response to chemotherapy, though the presence of hypoxic cells within the tumors likely remains a significant barrier to full resensitization of the tissue. A key to the success shown here seemed to be the determination of the appropriate conditions from studies using the engineered 3D tissue model which replicated the gradients in diffusible factors leading to tumor-derived quiescence (13, 16, 26, 34, 35). HCT116 and HT29 colorectal cell lines were chosen for this study as examples of related tumor types with differing levels of contact inhibition. HT29 cells express a 3-fold higher level of e-cadherin, a driver of contact inhibition of proliferation (36). HT29 cells have also been shown to be more resistant than HCT116 cells to strategies that use IGF-I receptor inhibition to control tumor cell growth (37). When grown as solid tumors, both cell types exhibited regions of chronic hypoxia in areas 100 to 150 μm from vessels, and both displayed a reduction in proliferation with distance from blood vessels. A comparison of these findings in other cancer types displaying both differing microenvironmental profiles and growth factor...
dependencies could help to determine the degree to which these IGF-I findings are widely applicable.

The 3D tissue model used here consisted of cancer cells grown into disks of tissue 30 to 40 cell layers thick to match typical diffusion distances seen in solid tumors (35, 38). Disks were grown in stirred media such that physiologic conditions were maintained on the surfaces of each disk while gradients in oxygen and nutrients formed with depth into the tissue from either surface. Under these conditions comparable gradients in proliferation were seen between the 3D tissue system and tumor xenografts. The initial screen using 3D-tissue disks showed that proliferation rates within tissue could be maintained by a combination of oxygen and supraphysiologic insulin or LR3-IGF-I supplementation. Their combined supplementation was found to reverse tumor-derived quiescence and resensitize the tissues to a panel of antiproliferative chemotherapies. Other diffusible molecules such as amino acids and glucose did not seem to limit proliferation when physiologic levels were maintained at the surface of the disks. However, supplementing glucose did increase the maximum depth that cells could survive to before becoming necrotic. Translation of in vitro findings from 3D tissue disks to the xenograft systems indicated a good correlation between the 2 models. In both cases moderate increases in proliferation status could be achieved via IGF-I receptor stimulation. Consistent with published data (39), the 5% oxygen exposure...
used for the 3D tissue disks appeared closest to actual tumor oxygen levels as in both cases pimonidazole staining increased dramatically 50 to 100 μm into the tissue. The presence of unmapped blood vessels lying outside of the tissue section may explain why this increase did not appear as sharp in the tumors compared with the 3D tissue disks. These unmapped vessels would act to skew the analysis of the tissue oxygenation in regions that seemed to be distant from mapped vessels but are actually close to unmapped ones. In mice, pure oxygen breathing at 1 and 2 atmospheres pressure did not alter hypoxia profiles or proliferation at the end of the 12-hour stimulation period. Hence the larger effects predicted from oxygen supplementation in disks could not be achieved in the xenografts. Working above 2 atmospheres oxygen was not feasible in mice due to their low tolerance compared with larger animals for prolonged exposures to hyperbaric oxygen and induction of pulmonary toxicity and bradycardia (40, 41).

Our results show that a significant resensitization of quiescent tumor cells to chemotherapy is achievable. A key step toward the successful translation of these findings may lie in follow-up studies to test hyperbaric oxygen strategies in larger animal models. In addition, the ability to stimulate proliferation on a repeating schedule may be highly dependant on the duration of activity of the antiproliferative drug combination that is being used. Hence, further work looking at a larger panel of drugs and over longer treatment schedules is necessary. Although a quiescent cell induction strategy has the potential to affect the response of any cancer, micrometastases might be a good target for future development as the inherent difficulty of their detection makes them less amenable to curative radiotherapy or surgery.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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