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Quantifying Transient Hypoxia in Human Tumor Xenografts by Flow Cytometry

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ABSTRACT

Transient hypoxia is a poorly understood and potentially important factor that may limit tumor response to various forms of therapy. We assessed transient hypoxia on a global scale in two different human tumor xenografts by sequentially administering two hypoxia markers followed by quantification of hypoxic cells using flow cytometry. High levels of the first hypoxia marker (pimonidazole) were maintained in the circulation over an 8-hour period by multiple hourly injections, providing a "time-integrated" hypoxia measure showing an asymptotic increase in the total number of hypoxic cells. Subsequent administration of a second hypoxia marker (CCI-103F) showed that substantial numbers of the previously pimonidazole-labeled cells were no longer hypoxic during the circulation lifetime of the second marker. The overall fraction of tumor cells that demonstrated changes in hypoxic status with time increased with different kinetics and by different magnitudes in the two xenograft systems. Specifically, up to 20% of the cells in SiHa (human cervical squamous cell carcinoma) tumors and up to 8% of the cells in WiDr (human colon adenocarcinoma) tumors were intermittently hypoxic over an 8-hour period. Also, the tumor cells that demonstrated transient hypoxia were typically not adjacent to functional tumor blood vessels. Similar approaches could be used in the clinic to provide information on the duration of intermittent hypoxia episodes and the fraction of transiently hypoxic tumor cells, which would, in turn, have important implications for the strategic improvement of cancer therapy.

INTRODUCTION

Many solid tumors contain subpopulations of cells that are poorly oxygenated, and the presence of these cells has been shown to limit the outcome of cancer therapy by radiation, chemotherapy, and even surgery (1). The oxygenation status of a tumor is now thought to have potential prognostic value in cancer treatment, and many methods for measuring tumor oxygenation have been developed. Exogenous markers such as pimonidazole (2) and EF5 (3) have been used clinically to label poorly oxygenated tumor cells for subsequent image analysis of tumor sections obtained by excision biopsy (4), for flow cytometry analysis of cells from excision or fine needle aspirate biopsies (5–7), or for visual scoring of tumor cell smears (8). However, although exogenous markers are useful tools to identify poorly oxygenated cells in both experimental and clinical tumors, a single injection or infusion of a marker (as is typically performed) only provides information on tumor oxygenation during the circulation lifetime of the marker. Moreover, only those cells that are at sufficiently low levels of oxygen for sufficient time intervals will bind measurable quantities of a given marker. Thus there are inevitable questions as to the therapeutic relevance of the labeled cells in terms of both the degree and duration of their oxygen deprivation.

A part of the ambiguity arises simply from terminology; hypoxia is, itself, a relatively ambiguous term. For the purposes of this manuscript, we use the terms "hypoxic" or "hypoxia" to denote oxygen deprivation that is sufficient to result in decreased sensitivity to ionizing radiation. Operationally, hypoxia markers have been designed to specifically label such cells (9, 10), and "thresholds" used to distinguish labeled from nonlabeled cells in flow cytometry analyses (11) provide tumor hypoxic fractions that are consistent with other functional assays such as the paired radiation survival curve technique (12). However, even though radiation survival curves provide unequivocal data regarding tumor radiosensitivity (and oxygenation indirectly), these measures are again assessed over the relatively short duration of the radiation exposure and therefore do not take into account changes in hypoxia that occur over time.

Tumor hypoxia was originally thought to arise exclusively in tumor cells situated at a substantial distance from functional tumor blood vessels ($>$100–150 μm), beyond which there is limited oxygen delivery due to the high oxygen consumption of the intervening cells (13). This chronic hypoxia has been extensively characterized in both animal and human tumor systems, and its impact on cancer therapy is well understood. However, observations in animal tumors more than two decades ago (14) led to suggestions of a more dynamic form of hypoxia, which has more recently been discussed as a potential factor impacting clinical cancer therapy (6, 7, 15, 16). This transient or intermittent hypoxia is poorly understood, and there are many unanswered questions regarding its presence and potential influence in the treatment of solid tumors. Transient hypoxia is thought to occur in tumor cells that are dependent on tumor blood vessels that are subject to partial and/or intermittent decreases in functionality, thereby reducing oxygen (and nutrient) delivery and availability. Importantly, as the oxygenation status of these cells increases or decreases over time, there are concomitant changes in their sensitivity to radiation (15) or chemotherapy (16). Moreover, a portion of transiently hypoxic cells are capable of retaining some proliferative capacity during the temporary reduction (or absence) of oxygen (11) and therefore could potentially proliferate upon reoxygenation of a tumor region. Thus although their presence in clinical tumors has been difficult to demonstrate conclusively thus far (largely due to inadequate methodology), transiently hypoxic tumor cells are potentially important players in limiting tumor response to therapy.

Efforts to better understand the mechanism of transient hypoxia generation have primarily involved monitoring changes in tumor oxygen tension (pO_2) over time by the insertion of one (or two) oxygen detection probes (with the presumption that the probe is positioned in a region of tumor containing viable cells). Oxygen tensions have been monitored continuously in murine tumors using recessed-tip oxygen microelectrodes (17–19) and in human melanoma xenografts using Oxylite fiber-optic microprobes (20, 21). These studies have provided real-time evidence that substantial yet highly heterogeneous changes in tumor blood flow and pO_2 can occur with different periodicities and magnitudes in different tumor types and in different regions of the same tumor. However, these data also reconfirm the spatial and temporal heterogeneity of the tumor microenvironment, suggesting the need for multiple measurements to more accurately depict the tumor as a whole. Moreover, the functional significance of temporal fluctuation in tumor pO_2 observed in this

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way is largely inferential because the overall fraction of viable tumor cells displaying a dynamic hypoxic status is not directly queried.

Transient hypoxia in human tumor xenografts has been indirectly studied in our laboratory via dual stain mismatch analyses of tumor sections, designed to observe microregional fluctuations in tumor blood flow. Differences between the relative staining intensities of two i.v. injected fluorescent dyes indicate tumor areas that were subject to perfusion changes during the time between stain injections (22–24). Presumably, regions of a tumor that are subject to fluctuations in blood flow (and thus oxygen delivery) may also exhibit changes in hypoxia depending on the degree and duration of the perfusion fluctuations. Although this technique is adequate for quantifying alterations in tumor perfusion in animal systems, there are technical and ethical limitations to extending the method to tumors in the clinic. Because information regarding the presence and duration of intermittent hypoxia episodes in clinical tumors would improve our understanding of the microenvironmental limitations of current cancer therapy protocols, an alternative approach for potentially measuring changes in tumor hypoxia in the clinic is needed.

To quantify changes in hypoxia over time and essentially over an entire tumor, we now report the use of sequential administration of the hypoxia markers pimonidazole and CCI-103F (25) in two different human tumor xenografts. This dual hypoxia marker strategy (26–28) with flow cytometry analysis allowed examination of hundreds of thousands of tumor cells, and those cells that contained one hypoxia probe and not the other indicate cells that were hypoxic at one point in time and "oxic" at another. A time-integrated tumor hypoxic fraction was estimated using hourly injections of pimonidazole, which could then be compared with the more "typical" hypoxic fraction determined with a single subsequent injection of CCI-103F. Importantly, the use of flow cytometry allowed quantification of transient hypoxia on a cell-by-cell basis, which provided an improvement in resolution when compared with the insertion of probes (17–21) or the analyses of tumor sections for changes in microregional perfusion (22–24) or hypoxia (4, 28). Moreover, flow cytometry analysis provides hundreds of thousands of data points for a single tumor, while also allowing the exclusion of necrosis and host cells from the measurements. Information on the proximity of transiently hypoxic cells relative to tumor vasculature that was functional just before tumor excision was obtained by sorting the tumor cells based on Hoechst 33342 perfusion (29, 30) before hypoxia marker analysis. Using an 8-hour observation window and two xenografted tumor systems, our data clearly indicate time- and tumor-dependent variation in the numbers and the proximity of transiently hypoxic cells to functional tumor vasculature. Furthermore, we found that hypoxia measurements that are "integrated" over time enable the inclusion of transiently hypoxic cells into estimates of tumor hypoxic fractions, and application of similar approaches in the clinic would allow assessment of the therapeutic relevance of these estimates.

MATERIALS AND METHODS

Tumors. Tumors derived from SiHa, a human cervical squamous cell carcinoma (31), and WiDr, a human colon adenocarcinoma (32), were used for all experiments. Both tumor cell types were obtained as cultured cell lines (American Type Culture Collection, Rockville, MD), grown in severe combined immunodeficient mice, and maintained by i.m. transplant. Experimental tumors were grown as dorsal s.c. implants in 7- to 8-week-old severe combined immunodeficient mice (bred in-house) and were used 3 to 4 weeks after implantation at an average tumor weight of ~500 mg. All procedures were performed in accordance with the ethical standards of the University of British Columbia Committee on Animal Care and the Canadian Council on Animal Care.

Reagents. Pimonidazole hydrochloride was supplied as Hydroxyprobe-1 (Natural Pharmaceuticals International, Inc., Belmont, MA), and CCI-103F was a generous gift from Dr. J. A. Raleigh (University of North Carolina, Chapel Hill, NC). Pimonidazole was dissolved at a concentration of 20 mg/mL in double-distilled water, and CCI-103F was dissolved in 10% DMSO and 90% peanut oil, also at 20 mg/mL. Both hypoxia probes were injected i.p. at a dose of 100 mg/kg mouse body weight. Pimonidazole and CCI-103F are conducive to combined assays for hypoxia because they have similar abilities to discriminate hypoxic cells, and neither inhibition of labeling nor cross-reactivity between primary antibodies has been observed in tumor section (28) or flow cytometry analyses (data not shown). Fluorescent activated cell sorting was based on the distribution of 1 mg of Hoechst 33342 (0.05 mL from a 20 mg/mL stock solution prepared in double-distilled water; Sigma-Aldrich Canada, Ltd., Oakville, Ontario, Canada) i.v. injected by the lateral tail vein 20 minutes before host sacrifice and tumor excision. Hoechst 33342 at this concentration was nontoxic to either host animals or tumor cells.

Cell Sorting and Flow Cytometry. Single-cell suspensions were prepared from excised experimental tumors by finely mincing with crossed scalpels and agitating the resulting brie for 40 minutes in an enzyme suspension containing 0.5% trypsin and 0.08% collagenase in Dulbecco's PBS (Gibco Invitrogen Corp., Burlington, Ontario, Canada). After incubation, 0.06% DNase was added, and the cell suspension was gently vortexed and filtered through 30-μm nylon mesh to remove clumps. Monodispersed cells were washed by centrifugation and resuspended in minimal essential medium (Gibco Invitrogen Corp.) containing 10% fetal bovine serum (HyClone, Logan, UT) for cell sorting (33, 34).

Viable tumor cells were sorted using a dual laser FACS 440 flow cytometer (Becton Dickinson, Mountainview, CA). Cells were defined on the basis of forward scatter (cell size); sort windows were automatically set to subdivide the cell population into six fractions of differing relative intracellular Hoechst 33342 concentrations. In general, the brightest Hoechst-stained cells, designated fraction 1, were proximal to functional vasculature, whereas the dimmest Hoechst-stained cells, designated fraction 6, were most distant from functional vasculature at the time of Hoechst injection (29, 30, 35).

Hoechst 33342 has a high avidity for DNA and slowly diffuses away from functional vasculature after i.v. injection, thereby establishing a marked gradient of staining intensity with increasing depth into tissue (29, 33). We have found a 20-minute interval between Hoechst injection and tumor excision provides an optimal Hoechst intensity gradient between brightly staining and dimly staining tumor cells with increasing distance from functional tumor blood vessels, thereby facilitating sorting based on the relative intracellular Hoechst intensity (29, 30, 35). The presence of cellular debris in the most dimly staining Hoechst fractions provides (indirect) evidence that the lower end of the Hoechst intensity gradient includes cells adjacent to tumor necrosis.

After the fluorescence-activated cell sorting was completed, the resultant tumor cell fractions were fixed in chilled 70% ethanol and stored at −20°C for a minimum of 24 h. Detection of intracellular hypoxia marker adducts was achieved by incubation with appropriate antibodies before analysis by flow cytometry (11). List mode files were collected using a dual laser Epics Elite-ESP flow cytometer (Coulter Corp., Hialeah, FL) and were subsequently reprocessed for analysis. Doublet correction and bitmap gating were used to select the cell populations of interest with the WINLIST software package (Verity Software House Inc., Topsham, ME). In addition, the diploid DNA content of the host (murine) normal cells enabled their exclusion from analysis of the hyperdiploid human tumor cells. Data derived from a combined analysis of pimonidazole and CCI-103F were normalized to the expected profile for simultaneous administration of the two markers (*i.e.*, close to 100% concordance between pimonidazole and CCI-103F–labeled cells). This normalization provided more conservative estimates of the fraction of cells labeled only with CCI-103F or only with pimonidazole.

Antibodies. Pimonidazole adducts were identified by an unconjugated IgG1 mouse monoclonal (clone 4.3.11.3) primary antibody (2) and a goat antimouse Alexa 594 fluorescent secondary antibody (Molecular Probes, Inc., Eugene, OR). CCI-103F adducts were visualized with anti-CCI-103F rabbit antisera (25) supplied by Dr. J. A. Raleigh and a polyclonal goat antirabbit Alexa 488 fluorescent secondary antibody (Molecular Probes, Inc.). DNA was counterstained with 4′, 6-diamidino-2-phenylindole dihydrochloride hydrate at a concentration of 2 μg/mL before flow cytometry analysis. All antibodies

were diluted in a solution containing 4% calf serum (HyClone) and 0.1% Triton X-100 in Dulbecco's PBS (Gibco Invitrogen Corp.).

RESULTS

Plasma pimonidazole levels were maintained at a high level over time by repetitively administering the hypoxia marker via hourly injections, enabling us to label hypoxic cells over a 1- to 8-hour period. For SiHa tumors, there was a statistically significant ($P < 0.05$) increase in the fraction of total tumor cells labeled with pimonidazole from 37% after a 1-hour period of pimonidazole labeling to 56% after 5 to 8 hours of exposure to pimonidazole (Fig. 1*A*).

We also examined pimonidazole labeling within subpopulations of SiHa tumor cells located at increasing distances from functional tumor blood vessels by sorting the cells based on Hoechst 33342 perfusion before flow cytometry analysis (Fig. 1*B*). As expected, there were an increasing number of pimonidazole-labeled cells as distance from tumor vasculature increased (decreased Hoechst staining intensity; higher sort fraction number). For example, with a single injection of pimonidazole (1 hour of labeling in Fig. 1*A*) and the tumor cells sorted into six fractions (Fig. 1*B*, open circles), we observed pimonidazole labeling in ~4% of the tumor cells staining most brightly with Hoechst (fraction 1 of 6; closest to functional vasculature). These cells correspond to ~0.7% of the total number of sorted tumor cells (4% × 1/6 of total tumor cells per sort fraction). Similarly, we observed that 72% of the cells furthest from functional tumor blood vessels were labeled with pimonidazole (fraction 6 of 6; 12% of the total number of tumor cells). Therefore, the increase in the total number of pimonidazole-labeled cells with time observed in Fig. 1*A* was primarily due to an increase in the number of pimonidazole-labeled cells found in sort fractions of intermediate to dim Hoechst intensity (fractions 3–6). Importantly, the increased total pimonidazole concentration administered over time did not induce observable nonspecific labeling of cells.

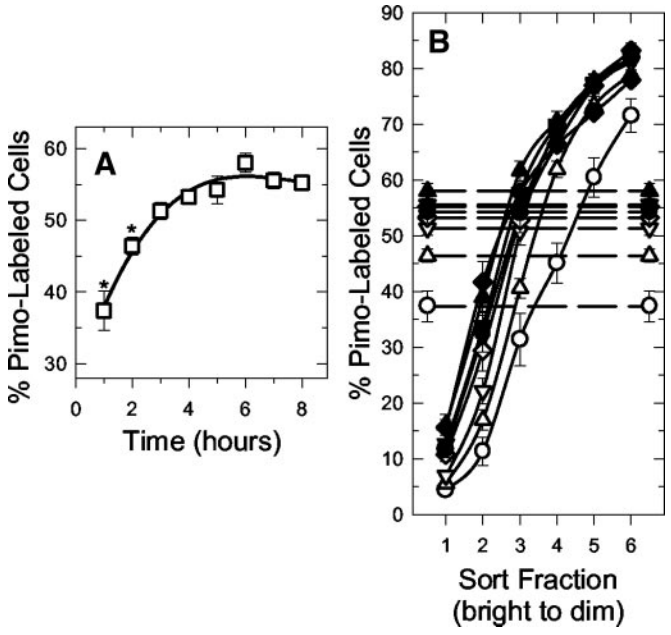

Fig. 1. Fraction of SiHa tumor cells labeled with pimonidazole (*Pimo*). *A*, percentage of Pimo-labeled tumor cells (□) after tumor-bearing mice were given hourly i.p. Pimo injections before tumor excision. *B*, same data with tumor cells sorted before Pimo analysis based on cellular content of the fluorescent perfusion dye Hoechst 33342. *Dashed lines*, percentage of Pimo-labeled cells from whole tumors. Mice were given hourly Pimo injections for 1 hour (○), 2 hours (△), 3 hours (▽), 4 hours (◇), 5 hours (●), 6 hours (▲), 7 hours (▼), or 8 hours (◆). Data are mean; $n = 4$–9 tumors; *bars* are ±SE. ∗, statistically significant difference with each subsequent time point ($P < 0.05$).

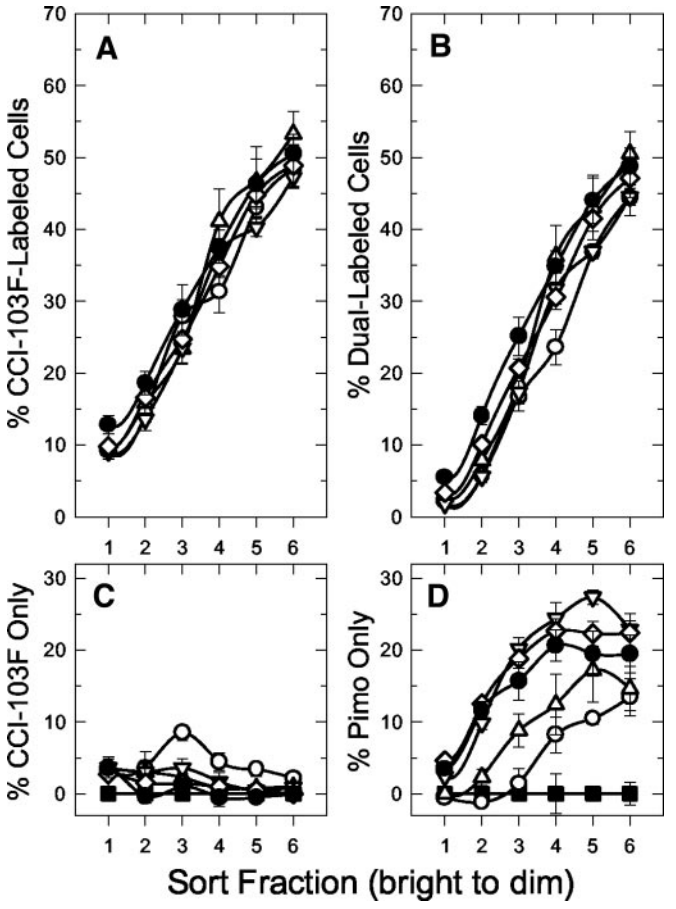

Fig. 2. Fraction of sorted SiHa tumor cells labeled with CCI-103F and/or pimonidazole (*Pimo*). *A*, percentage of tumor cells labeled with CCI-103F 2 hours after i.p. injection of the marker. *B*, percentage of SiHa tumor cells labeled with both Pimo and CCI-103F. *C*, percentage of SiHa tumor cells labeled with CCI-103F but not previously labeled with Pimo. *D*, percentage of SiHa tumor cells labeled with Pimo but not labeled with subsequent CCI-103F. Mice were given hourly Pimo injections 1 hour (○), 2 hours (△), 3 hours (▽), 4 hours (◇), or 5 hours (●) before CCI-103F injection or a single Pimo injection simultaneously with CCI-103F (■). Curves for six to eight hourly Pimo injections were not significantly different than the 5-h curve and were omitted for clarity. Data are mean; $n = 4$–6 tumors; *bars* are ±SE.

We injected a second hypoxia marker, CCI-103F, 1 hour after the final pimonidazole injection and 2 hours before tumor excision for each of the tumors (Fig. 2*A*). The fraction of tumor cells labeled with both pimonidazole and subsequent CCI-103F is shown in Fig. 2*B*, indicating cells that were sufficiently hypoxic at some point during the circulation lifetimes of each hypoxia probe to label with both markers. It is worth noting that although these cells were "labeled" with both hypoxia markers, they may or may not have been hypoxic for the entire labeling duration of either marker. In addition, the degree of hypoxia as assessed by CCI-103F labeling did not increase with increased pimonidazole administration, indicating that the constant labeling of hypoxic cells with pimonidazole did not affect tumor oxygenation (10).

Fig. 2*C* and *D* indicate the percentage of tumor cells in each sort fraction that were exclusively labeled with CCI-103F or pimonidazole, respectively. Thus the total fraction of CCI-103F–labeled SiHa cells shown in Fig. 2*A* included cells dual-labeled with pimonidazole and CCI-103F (Fig. 2*B*) and cells labeled only with CCI-103F (Fig. 2*C*). Similarly, the total fraction of pimonidazole-labeled SiHa cells in Fig. 1 included cells dual-labeled with pimonidazole and CCI-103F (Fig. 2*B*) and cells labeled only with pimonidazole (Fig. 2*D*). Importantly, the profiles in Fig. 2*D* represent cells that were hypoxic enough during the pimonidazole exposure to label with that marker but were

not hypoxic enough during the subsequent exposure to CCI-103F to label with that marker. As the duration of pimonidazole exposure was increased, there was an increase in the fraction of cells that labeled only with pimonidazole. These data suggest that the increase in total pimonidazole-labeled cells with time observed in Fig. 1*A* was due to the detection of these cells that, by definition, were transiently hypoxic. The relative number of cells that were transiently hypoxic ranged from 5 to 20% of the total tumor cells during the 1- to 5-hour period of pimonidazole labeling. Interestingly, the majority of cells that demonstrated changes in hypoxia over this time period were located at an intermediate to large distance from tumor vasculature that was functional just before tumor excision (sort fractions 3–6).

Typical flow cytometry profiles are presented in Fig. 3 for comparison of a SiHa tumor in which pimonidazole and CCI-103F were administered simultaneously (Fig. 3*A* and *B*) and a SiHa tumor with five hourly injections of pimonidazole followed by an injection of CCI-103F (Fig. 3*C* and *D*). Tumor cells were sorted based on Hoechst 33342 perfusion before flow cytometry analysis and profiles for the brightest and the dimmest Hoechst-stained sort fractions are shown in each case. For all plots, pimonidazole-positive cells are above the horizontal threshold and CCI-103F–positive cells are to the right of the vertical threshold; the dashed diagonal line is included as an approximate slope of the population of dual-labeled cells. The majority of hypoxic tumor cells in Fig. 3*A* and *B* are dual-labeled with few single-labeled cells (labeled only with pimonidazole or CCI-103F). Cells labeled only with pimonidazole are observed in Fig. 3*C* and *D* as upward shifts of the dual-labeled populations from the unlabeled cells due to an increase in the fraction of cells above the (horizontal) threshold for being considered pimonidazole positive, and to the left of the (vertical) threshold and therefore CCI-103F negative.

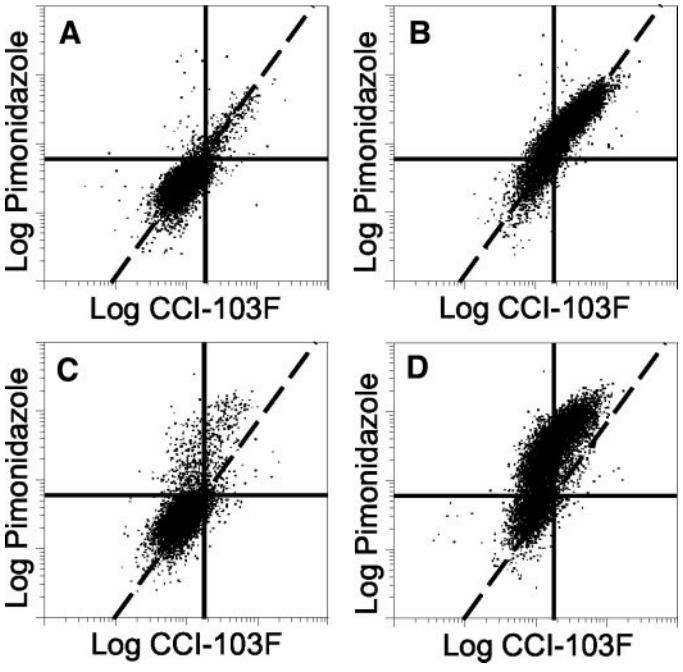

Fig. 3. Representative flow cytometry dot plots showing hypoxic SiHa tumor cells labeled with pimonidazole and CCI-103F. The threshold (*solid*) lines on each plot are for illustration purposes only; actual thresholds used for analysis were set using linear distributions of each hypoxia marker *versus* DNA content and gating on multivariate plots. Sort fraction 1 (*A*; brightest Hoechst) and sort fraction 6 (*B*; dimmest Hoechst) for SiHa tumor with pimonidazole and CCI-103F administered simultaneously 2 hours before tumor excision. Sort fraction 1 (*C*) and sort fraction 6 (*D*) for SiHa tumor with five hourly injections of pimonidazole followed by a single CCI-103F injection 2 hours before tumor excision. Note that the distribution of labeled cells has a distinctly different shape (upward shift relative to the *dashed line*) due to the increased fraction of cells labeled only with pimonidazole.

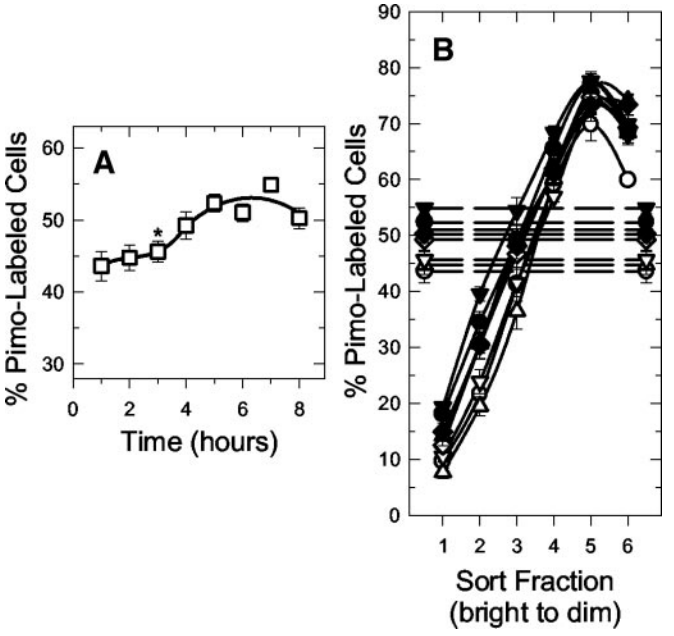

Fig. 4. Fraction of WiDr tumor cells labeled with pimonidazole (*Pimo*). *A*, percentage of Pimo-labeled tumor cells (□) after tumor-bearing mice were given hourly i.p. Pimo injections before tumor excision. *B*, same data with tumor cells sorted before Pimo analysis. *Dashed lines*, percentage of Pimo-labeled cells from whole tumors. Mice were given hourly Pimo injections for 1 hour (○), 2 hours (△), 3 hours (▽), 4 hours (◇), 5 hours (●), 6 hours (▲), 7 hours (▼), or 8 hours (◆). Data are mean; $n = 4–8$ tumors; *bars* are ±SE. *, statistically significant differences between each preceding time point and the 5-h point ($P < 0.05$).

Fig. 3 also effectively illustrates the benefits of flow cytometry for quantifying the continuum of hypoxic status for tens of thousands of tumor cells simultaneously. Our approach provides a vastly increased number of discrete measurements and an improved resolution when compared with regional pO_2 detection with solid probes (17–21) or microregional hypoxia marker detection in tumor sections (4, 28). Moreover, the differences in flow cytometry profiles between cells of different Hoechst intensities visually reinforce the relative rarity of observing hypoxic cells directly adjacent to tumor blood vessels that were functional just before analysis (which would be indicative of relatively large-scale changes in vascular functionality).

For WiDr tumors, there was a statistically significant ($P < 0.05$) increase in the fraction of total tumor cells labeled with pimonidazole from 44% after 1 to 3 hours to 52% by 5 to 8 hours (Fig. 4*A*). As with the SiHa experiments, we sorted the tumor cells based on Hoechst perfusion before flow cytometry analysis (Fig. 4*B*) and observed a less dramatic increase in pimonidazole-labeled cells with time. Interestingly, there was a decrease in pimonidazole labeling in the most dimly Hoechst-stained cells (sort fraction 6) for all WiDr curves relative to the preceding sort fraction that was not observed in SiHa tumors. Because pimonidazole preferentially labels viable hypoxic cells (11), this disparity may be due to a decrease in WiDr tumor cell viability at a large distance from tumor vasculature. Indeed, these tumors typically contain relatively large areas of necrosis away from tumor blood vessels and a large amount of cellular debris, suggesting a high degree of cell mortality in these regions. Conversely, we have found that SiHa tumors tend to contain far less cellular debris and that a larger fraction of viable SiHa cells are recoverable per gram of tumor when compared with WiDr tumors.

When CCI-103F was administered to WiDr tumors 1 hour after the final pimonidazole injection and 2 hours before tumor excision (Fig. 5*A*), the decrease in pimonidazole labeling in the most dimly Hoechst-stained fraction was also observed for CCI-103F. This is

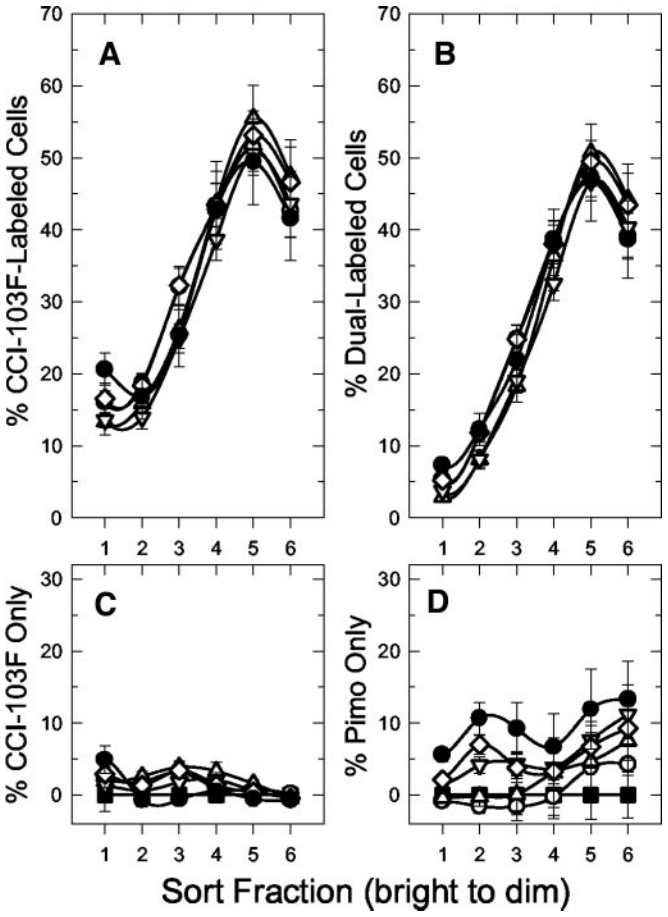

Fig. 5. Fraction of sorted WiDr tumor cells labeled with CCI-103F and/or pimonidazole (*Pimo*). *A*, percentage of tumor cells labeled with CCI-103F 2 hours after i.p. injection of the marker. *B*, percentage of WiDr tumor cells labeled with both Pimo and CCI-103F. *C*, percentage of WiDr tumor cells labeled with CCI-103F but not previously labeled with Pimo. *D*, percentage of WiDr tumor cells labeled with Pimo but not labeled with subsequent CCI-103F. Mice were given hourly Pimo injections for 1 hour (○), 2 hours (△), 3 hours (▽), 4 hours (◇), or 5 hours (●) before CCI-103F injection, or a single Pimo injection simultaneously with CCI-103F (■). Curves for six to eight hourly Pimo injections were not significantly different than the 5-h curve and were omitted for clarity. Data are mean; $n = 4–8$ tumors; *bars* are ±SE.

consistent with the previous explanation (*e.g.*, decreased cell viability), independent of the individual hypoxia marker used. Additionally, there was a relatively high level of CCI-103F labeling in the brightest Hoechst staining fraction (sort fraction 1) compared with the pimonidazole labeling in Fig. 4*B* for the same subpopulation of cells.

Tumor cells that were labeled with both pimonidazole and subsequent CCI-103F are indicated in Fig. 5*B*, and tumor cells that were labeled either only with CCI-103F or only with pimonidazole are indicated in Fig. 5*C* and *D*, respectively. Although there is an observable increase in the fraction of cells labeled only with pimonidazole over time (Fig. 5*D*), the differences between the curves are much less distinct than for the SiHa data (Fig. 2*D*). Up to 8% of the total number of unsorted WiDr tumor cells were transiently hypoxic over the 5-hour period, with an approximately equal contribution from cells in each sort fraction for the 3- to 5-hour curves.

DISCUSSION

Our previously published dual stain mismatch data (7) indicated that SiHa and WiDr tumor xenografts have very different propensities to undergo fluctuations in tumor perfusion over a 30-minute period. Changes in WiDr perfusion (and likely hypoxia) typically occur with a frequency of less than 1 hour, and these perfusion changes can be quite dramatic in some tumor regions representing up to 8-fold differences in the delivery of two sequentially administered fluorescent perfusion dyes. Conversely, SiHa perfusion tends to be much more stable over relatively short time intervals, and perfusion changes (when present) are typically of a lesser magnitude. These observations had previously led us to believe that SiHa tumor blood flow was fairly constant and that there was little transient hypoxia in these tumors. Although this statement remains true when considered on the order of minutes, the data reported herein suggest that substantial changes in the hypoxic status of SiHa tumor cells can occur over several hours.

Quantification of transiently hypoxic cells in solid tumors has a number of practical implications for the clinic. Of primary importance is the ability to detect these cells, and to address whether the presence of intermittent hypoxia correlates with poor clinical prognosis. From a therapy standpoint, the presence and fraction of transiently hypoxic cells in a tumor could conceivably dictate the type of treatment strategy that is used (35). For example, strategies that improve tumor perfusion (36) or that involve repeated low doses of radiation (15) or drugs (16) would be expected to target cells with changing hypoxic status. Ideally, quantification of the intermittently hypoxic content of a tumor could be used to individualize treatments designed to target hypoxic tumor cells.

Hypoxia probes such as pimonidazole "integrate" tumor hypoxia over the circulation time of the agent (murine plasma elimination $t_{1/2} = 30$ minutes; ref. 37). Thus a single 100 mg/kg injection of pimonidazole would label cells that were hypoxic enough (for long enough) during a ~1-hour period without distinguishing between cells that were hypoxic for all or part of this time. For experimental tumors that exhibit changes in tumor perfusion (and likely hypoxia) over periods of less than 1 hour such as WiDr tumors, pimonidazole labeling would not detect minor or short-lived changes in hypoxia that occurred within this time frame. This is important insofar as the estimates for the fraction of transiently hypoxic cells included in this manuscript should be viewed as conservative (particularly for WiDr tumors) because they do not take into consideration changes in tumor hypoxia that may have occurred with a duration of less than 1 hour. Similarly, our previously published data indicating changes in SiHa tumor hypoxia over many days using time-integrated orally administered pimonidazole (38) should also be viewed as conservative (where the shortest interval of hypoxia marker labeling was 6 h).

Although we did not attempt to shorten the interval of hypoxia labeling with pimonidazole, we were able to increase it by performing multiple hourly injections of the marker. SiHa tumors demonstrated distinct hypoxia marker "mismatch" when pimonidazole exposure was maintained, indicating the number of hypoxic tumor cells increased over times up to at least 5 h. Importantly, the fraction of SiHa tumor cells that were hypoxic over a 1-hour period was 37% (determined by a single pimonidazole injection as is typically performed in rodents), but increased significantly to 56% when considered over a longer period of time. This increase in the total fraction of hypoxic cells was due to the inclusion of cells that were not labeled with subsequent CCI-103F and were therefore transiently hypoxic. Thus the time-integrated administration of pimonidazole over a 5-hour period provided an estimate for the hypoxic fraction of SiHa tumors that included cells that were transiently hypoxic.

WiDr tumors demonstrated a large degree of concordance between the two hypoxia markers over the same time frame. Indeed, the fraction of hypoxic cells increased only marginally from 44 to 52% from 1 to 5 hours of pimonidazole exposure, indicating a lesser propensity for WiDr tumors to undergo changes in hypoxia over this time period. When combined with the dual stain mismatch data indicating a large degree of perfusion fluctuation in WiDr tumors over

a 30-minute interval (7), these data suggest that the bulk of intermittent hypoxia episodes in WiDr tumors occur with a frequency of less than 1 hour. Therefore, a single pimonidazole injection appears to provide a hypoxic fraction that includes the majority of transiently hypoxic cells in WiDr tumors. A more accurate representation of transient hypoxia (specifically) in WiDr tumors would involve assessing differences in tumor hypoxia over shorter time intervals, perhaps combining time-integrated pimonidazole labeling with differential quantification of a short-lived endogenous hypoxia "marker" such as HIF-1α (39–41).

The intratumoral proximity of transiently hypoxic tumor cells relative to vasculature that was perfused just before tumor excision was also determined. Transiently hypoxic cells in SiHa tumors were found primarily at an intermediate to large distance from functional tumor vasculature, which is an observation that has important mechanistic implications. With our original dual stain mismatch data indicating relatively large perfusion changes in (some) tumor blood vessels (22), it was tempting to assume that transient hypoxia was largely a result of complete stoppages of flow through tumor blood vessels. Although larger scale changes in perfusion are the most impressive to observe, they have perhaps taken attention away from more modest (and more frequently observed) changes in perfusion that may also impact tumor hypoxia (23, 24). Indeed, other studies monitoring erythrocyte flux through tumor blood vessels in murine tumors have shown that modest changes in flux are more common than complete stoppages in tumor blood flow (42).

The location of transiently hypoxic cells at an intermediate to large distance from tumor vasculature makes intuitive sense when one considers the location of the boundary between "oxygenated" (*i.e.*, radiosensitive) and radiobiologically hypoxic cells in relation to functional blood vessels. For changes in tumor perfusion to impact the oxygenation of cells directly adjacent to a functional blood vessel, the vessel would have to experience a drastic decrease in flow (*i.e.*, complete collapse or obstruction). However, a much less drastic decrease in blood vessel functionality could conceivably shift the boundary between "oxic" and hypoxic cells toward the vessel by a few cell diameters. The oxygenation of cells at an intermediate distance from tumor blood vessels should therefore be more susceptible to modest blood flow changes than the cells directly adjacent to tumor vasculature. Due to the radial geometry of tumor cords surrounding tumor blood vessels, the number of cells that could potentially be affected by modest changes in tumor perfusion could conceivably be quite large. Thus the number of tumor cells affected by (relatively infrequent) large-scale changes in tumor perfusion is likely small relative to the number of cells potentially affected by more modest perfusion changes. Measuring changes in tumor hypoxic status on a cellular level using flow cytometry analysis provides a means to detect and quantify these cells, with a resolution that is not possible with regional pO_2 observation (17–21) or microregional hypoxia marker detection (4, 28).

It is worth noting that SiHa tumor cells that demonstrated changes in their hypoxic status over time were present in a similar proximity to tumor blood vessels as cells inferred to be at a static level of intermediate oxygenation (43–45). Additional work is required to resolve these two conceptually different (though possibly related) types of hypoxic cell, although both are potentially important limitations of cancer therapy.

Because some tumor cells may cycle in and out of hypoxia over time, assessing tumor hypoxia over relatively short periods of time likely underestimates the true (potentially therapeutically relevant) hypoxic fraction of a tumor. Thus the administration of hypoxia markers such as pimonidazole by a single i.p. injection in experimental animals or by i.v. infusion in patients [plasma half-lives of 30 minutes (37) and 5.1 hours (46), respectively] may not provide an accurate picture of tumor hypoxia when considered over a period of many hours in rodents or a day or more in patients. Changes in hypoxia over longer time intervals may not be considered important when administering a single dose of therapy but could potentially have a substantial impact on tumor response to multi-fractionated radiotherapy (15) or chemotherapy (16). Changes in tumor hypoxia over time may also affect the regrowth potential of a tumor as formerly hypoxic cells may suddenly find themselves in an environment that is better oxygenated and more favorable for proliferation (6, 11). Moreover, an additional complexity arises when one considers changes in tumor hypoxia that may be induced by the tumor response to therapy itself, a factor that has not been adequately studied in the clinic to this point. Clearly, a method for measuring changes in tumor hypoxia over time may have application to determining treatment-induced changes in tumor hypoxia, whereas time-integrated hypoxia measurements may provide more therapeutically relevant estimates of hypoxia before therapy.

By using two sequentially administered hypoxia markers with subsequent analysis by flow cytometry, we have established the presence and influence of intermittent hypoxia episodes on a whole-tumor scale and provided estimates of the transiently hypoxic fractions of two human tumor xenografts. A similar method (*e.g.*, using pimonidazole and EF5) may be applicable to determining the presence and influence of transiently hypoxic cells in biopsies obtained from human tumors in the clinic. Once these critical clinical questions have been addressed, it follows that re-evaluation of methods used to measure hypoxia in the clinic may be necessary. Overall, improving our understanding of transient hypoxia will aid in the design of clinical cancer therapy protocols to take advantage of the dynamic nature of the solid tumor microenvironment.

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