Orally Administered Pimonidazole to Label Hypoxic Tumor Cells

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Abstract

Pimonidazole, a “hypoxia marker” normally delivered i.v. or i.p., was instead administered in the drinking water of tumor-bearing mice. As pimonidazole exposure was increased from 3–96 h ad libitum, both the fraction of hypoxic tumor cells and the relative number of pimonidazole adducts in those cells increased. Furthermore, the sustained ingestion of pimonidazole revealed a larger hypoxic fraction than did a single injection of an alternative hypoxia marker, CCI-103F. The “additional” hypoxia seen with longer-term oral administration apparently reflects the inclusion of transiently hypoxic tumor cells. Thus, in addition to its convenience and versatility when compared with hypoxia marker injection, oral administration of pimonidazole appears to permit identification of all of the physiologically and therapeutically relevant hypoxic tumor cells.

Introduction

Poorly oxygenated cells in most solid tumors profoundly decrease curability by radiation, chemotherapy, and even surgery (1). Considerable effort has consequently been devoted to developing techniques for quantifying tumor hypoxia in patients. Unfortunately, all of the current methods are inconvenient, requiring the insertion of a polarographic or fiber-optic probe (2, 3), irradiation immediately following by “comet assay” of cells removed by fine-needle aspiration (4), or injection of a “hypoxia tracer” with regional imaging (5) or biopsy if microregional information is desired (6–8). Furthermore, these techniques provide rather static information, encompassing only the time the electrode is in place, the interval over which radiation is delivered, or the circulating lifetime of the injected hypoxia marker. With these limitations in mind, we discovered that the commonly used hypoxia marker pimonidazole (9) provides useful and novel information when administered p.o. in the drinking water of tumor-bearing mice. Furthermore, a quite different picture of tumor hypoxia emerges when pimonidazole is administered over longer periods of time, both as a single agent and when compared with an injection of a different hypoxia marker, CCI-103F (10), in our tumor model. Consequently, p.o. administered pimonidazole not only adds convenience and versatility to the measurement of tumor hypoxia, a variable with increasingly recognized prognostic value for cancer therapy, but also has the potential to provide new insights into tumor dynamics.

Materials and Methods

Reagents. Pimonidazole hydrochloride was purchased as Hydroxyprobe-1 (NIPI, Inc., Belmont, MA), and CCI-103F was synthesized in the laboratory of J. A. R. (10). Pimonidazole was dissolved at a concentration of 1 mg/ml in sterilized water for oral administration; CCI-103F was dissolved in 10% DMSO plus 90% peanut oil at a concentration of 20 mg/ml for i.p. injection. Pimonidazole and CCI-103F are conductive to combined assays for hypoxia because they label comparable fractions of hypoxic cells, and neither inhibition of labeling nor cross-reactivity between primary antibodies has been observed in tumor section (11) or flow cytometry analyses (data not shown). Cell sorting was based on the distribution of 1 mg 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 min before host sacrifice and tumor excision (12). Hoechst 33342 at this concentration was nontoxic to either host animals or tumor cells.

Tumors. Tumors derived from SiHa, a human cervical squamous cell carcinoma (13), were used for all of the experiments. The tumor cells were obtained as a cultured cell line (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 7–8 week old male severe combined immunodeficient mice (bred in-house), and were used 3–4 weeks after implantation at an average weight of ～500 mg. All of the 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. Single-cell suspensions were prepared from experimental tumors by excising the tumor, then finely mincing with crossed scalpels and agitating the resulting brei for 40 min in an enzyme suspension containing 0.5% trypsin and 0.08% collagenase in Dulbecco’s PBS (Invitrogen Corp., Burlington, Ontario, Canada). After incubation, 0.06% DNase was added, and the cell suspension gently vortexed and filtered through 30-μm nylon mesh to remove clumps. Monodispersed cells were washed by centrifugation and resuspended in minimal essential medium (Invitrogen Corp.) containing 10% fetal bovine serum (HyClone, Logan, UT) for cell sorting (14).

Cell Sorting and Flow Cytometry. Viable tumor cells, prepared as described above, were sorted using a dual laser FACS 440 (Becton Dickinson, Mountain View, CA). Cells were defined on the basis of forward scatter (cell size); sort windows were automatically set to subdivide the cell population into the desired fractions of differing intracellular Hoechst concentrations (14). The brightest Hoechst-stained cells, designated fraction 1, were proximal to functional vasculature, whereas the dimmest Hoechst-stained cells, designated fraction 6, were distant from functional vasculature (12). After the fluorescence-activated cell sorting was completed, the resultant tumor cells were fixed in chilled 70% ethanol. Detection of intracellular hypoxia marker adducts was achieved by incubation with appropriate antibodies before analysis by flow cytometry (15). 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). Additionally, the diploid DNA content of the host (murine) normal cells enabled their exclusion from analysis of the hyperdiploid human tumor cells.

Antibodies. Adducts of pimonidazole were identified by an unconjugated IgG1 mouse monoclonal primary antibody (clone 4.3.11.3; Ref. 9) and a monoclonal Alexa 594 fluorescent secondary antibody (Molecular Probes Inc., Eugene, OR). CCI-103F adducts were visualized by anti-CCI-103F rabbit antisera (10) and a polyclonal goat anti-rabbit Alexa 488 fluorescent secondary antibody (Molecular Probes Inc.). DNA was counterstained with 4’,6-diamidino-2-phenylindole at a concentration of 1 μg/ml before flow cytometry analysis. All of the antibodies were diluted in a solution containing 4% bovine calf serum (HyClone) and 0.1% Triton X-100 in Dulbecco’s PBS (Invitrogen Corp.).

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Results and Discussion

Fraction of Tumor Cells Labeled with Oral Pimonidazole. Recognizing the limited information obtainable with a single injection of a hypoxia marker and the impracticability of multiple i.v. injections, we gave pimonidazole p.o. (ad libitum) to SiHa tumor-bearing mice for 3–96 h and measured the fraction of tumor cells that had incorporated the marker (Fig. 1A). Interestingly, we observed a rapid increase in the fraction of pimonidazole-labeled cells up to 12 h (Fig. 1A, dashed line), followed by an additional gradual increase in labeling up to 96 h (Fig. 1A, solid line). For reference, the 6-h time point identified a hypoxic fraction quite comparable with that observed in previous studies with SiHa tumors when a single i.v. or i.p. injection of pimonidazole and an equally rigorous definition of labeling were used (15). Consequently, the substantial increase in hypoxic fraction observed between 6 and 12 h with continued oral administration of pimonidazole was unexpected.

As shown in Fig. 1B, we also evaluated oral pimonidazole labeling within fractions of tumor cells located at various distances from tumor blood vessels (12) using fluorescence-activated cell sorting based on intracellular content of the vital DNA dye Hoechst 33342 (14). By essentially “breaking down” tumors into cell subpopulations from regions of differing blood flow (at the time of Hoechst administration), we were able to observe substructure in the pimonidazole labeling. Specifically, there was an increased percentage of pimonidazole-labeled cells with increasing distance from functional tumor vasculature (decreased Hoechst staining intensity; dimmer sort fraction). Moreover, the increased number of pimonidazole-labeled cells observed beyond 6 h in Fig. 1A was largely because of labeling of cells that were not directly adjacent to tumor blood vessels functional just before tumor excision and assay.

Relative Fluorescence Intensity of Tumor Cells Labeled with Oral Pimonidazole. By assessing the fluorescence intensity of pimonidazole labeling as a function of cell location relative to functional vasculature (sort fraction), we also identified a rather unexpected pattern (Fig. 2A). For those cells that incorporated pimonidazole (recognizing that many fewer pimonidazole-labeled cells were in fact near blood vessels), we found that cells at an intermediate radial distance from tumor vasculature demonstrated the least pimonidazole binding. This presumably reflects, in part, that cells nearer tumor blood vessels were more likely to be proliferating and, thus, have a larger volume than cells that were further from functional blood vessels. Therefore the larger, presumably more metabolically active cells adjacent to functional blood vessels tended to be more intensely labeled with pimonidazole when hypoxic. With sustained hypoxia, the smaller cells that were more distant from tumor vasculature became more intensely labeled than the intermediate cells. Taken together, these data illustrate a rather complex interplay between pimonidazole delivery/binding and tumor cell proximity to functional vasculature.

We also examined the relative fluorescence intensity of pimonidazole labeling in the least-well-perfused cells (fraction 6) and compared this with the estimated pimonidazole exposure based on the volume of water consumed as shown in Fig. 2B. It was found that the amount of incorporated pimonidazole per labeled cell progressively increased with time, essentially paralleling pimonidazole exposure. Again, it should be noted that both the number of pimonidazole-labeled cells (Fig. 1A) and the intensity of pimonidazole per labeled cell (Fig. 2B) increased with time.

Comparison of Oral Pimonidazole and CCI-103F. To clarify the dynamics of hypoxia labeling by oral pimonidazole, a second hypoxia marker, CCI-103F (10), was injected i.p. 3 h before tumor excision. Thus CCI-103F binding provided a relative “snapshot” of tumor hypoxia over 3 h, whereas oral pimonidazole “integrated” hypoxia over a longer period of time. The percentage of tumor cells labeled with CCI-103F in various SiHa tumors was not significantly different.
Mice were allowed to drink Pimo-containing water for 3 h (including cells labeled only with Pimo, and cells dual labeled with Pimo and CCI-103F. Thus, the total fraction of Pimo-labeled cells labeled with subsequent i.p. CCI-103F. Thus, the total fraction of Pimo-labeled cells labeled with pimonidazole but did not label with CCI-103F, we were able to detect almost twice the number of hypoxic tumor cells as compared with the snapshot of tumor hypoxia given by short-term administration! These data raise the (probably alarming) suggestion that methods of assessing tumor hypoxia over relatively short periods of time might underestimate the therapeutically relevant hypoxic fraction of a tumor.

There is some clinical evidence to support preclinical studies suggesting that tumors can contain regions with temporally changing blood flow and oxygenation (17, 18). Consequently, the possibility that current clinical methods to measure tumor oxygenation are unable to identify tumor cells that cycle in and out of hypoxia with time should not be ignored. Such changes may not be considered important when administering a single dose of therapy, but would very likely have a significant impact on tumor response to and tumor regrowth during (15, 17) multifraction chemotherapy (18) or radiotherapy (19, 20). Therefore, hypoxic fractions determined over relatively longer periods of time may prove more relevant over a course of therapy. Through the novel use of the well-established hypoxic cell marker pimonidazole, we believe that improved estimates of physiologically and therapeutically relevant tumor hypoxia can be achieved.

Oral administration of pimonidazole offers the primary advantages of convenience and versatility in the determination of clinical tumor hypoxia. Implicit in this statement is the potential to determine the hypoxic fraction of a tumor not only before therapy, but also during treatment in hopes of identifying those tumors that are responding poorly. Pimonidazole is more convenient to administer p.o. rather than by infusion, and quantification of hypoxia in clinical tumors can be minimally invasive with analysis of fine needle aspirate biopsies by image or flow cytometry (17). Moreover, the labeling pattern of oral pimonidazole provides an integrated hypoxic fraction over longer time periods than hypoxia marker injection, allowing the inclusion of tumor cell populations that exhibit transient changes in hypoxia into measurements of tumor hypoxia. Many groups have suggested that these transiently hypoxic cells may be the most therapeutically relevant hypoxic cells (19, 20) because they are thought to retain proliferative capacity (15) while showing the treatment resistance of hypoxic cells. With oral delivery of pimonidazole, this critical clinical question may finally be addressed.

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References


Fig. 3. Fraction of tumor cells labeled with CCI-103F and/or pimonidazole (Pimo). A, percentage of tumor cells labeled with CCI-103F 3 h after i.p. injection of the marker. Tumor cells were sorted before analysis. B, percentage of tumor cells labeled with both oral Pimo and i.p. CCI-103F. C, percentage of tumor cells labeled with oral Pimo but not labeled with subsequent i.p. CCI-103F. Thus, the total fraction of Pimo-labeled cells was able to identify and quantify cells that were subject to alterations in hypoxic status (Fig. 3). That is, we documented tumor cells that were sufficiently hypoxic during the long-term exposure to oral pimonidazole to incorporate that marker, yet were not hypoxic enough by CCI-103F binding. By analyzing the fraction of tumor cells that were able to incorporate CCI-103F during the 3 h just before analysis. Importantly, pimonidazole and CCI-103F have been shown to label comparable fractions of hypoxic tumor cells in both solid tumors (11) and spheroids (data not shown). These data suggest that the differences in pimonidazole labeling observed between 6 and 12 h of pimonidazole exposure may be because of transient changes in tumor cell oxygenation, and that a small fraction of cells were subject to even longer-term changes in oxygenation. As the timing of blood flow changes will undoubtedly be quite different in different tumor systems, oral administration of pimonidazole should provide a more convenient way of making such determinations than the multiple injections of the marker used previously to study tumor blood flow dynamics (17). Fig. 3 also reinforces the suggestion that increases in hypoxia measured by long-term pimonidazole exposure were not because of any adverse effects of oral pimonidazole on oxygen supply to the tumors (16). Importantly, the hypoxic fraction of ~500 mg SiHa tumors was ~15% when observed either with CCI-103F or with pimonidazole over a 6-h period, but was close to 30% when pimonidazole was delivered for ≥12 h. Thus, when the measurement of tumor hypoxia was integrated over time through the use of oral pimonidazole, we were able to detect almost twice the number of hypoxic tumor cells as compared with the snapshot of tumor hypoxia given by short-term administration! These data raise the (probably alarming) suggestion that methods of assessing tumor hypoxia over relatively short periods of time might underestimate the therapeutically relevant hypoxic fraction of a tumor.

over a 3-h period, and, of particular importance, ingestion of pimonidazole did not adversely affect tumor oxygenation (16) as assessed by CCI-103F binding (Fig. 3A). During a simultaneous 3-h exposure to pimonidazole (oral; 1 mg/ml) and CCI-103F, tumor hypoxia was detectable by CCI-103F but only minimally by pimonidazole binding as expected with the relatively low dose of pimonidazole consumed over this time. However, by 6 h, oral pimonidazole produced very similar information to that obtained from the 3-h exposure to CCI-103F as shown in Fig. 3B. Importantly, these results argue that the differences in labeling observed between 12 and 6 h of oral pimonidazole exposure shown in Fig. 1 were not because of inadequate pimonidazole ingestion or binding at 6 h. Taken together, these data suggest that any potential decrease in binding of p.o. administered pimonidazole induced by pharmacokinetic limitations (including drug metabolism) were minimal with 6 h of ad libitum consumption. However, the actual dose of pimonidazole received by the tumor is unknown, and more rigorous pharmacokinetic studies of p.o. administered pimonidazole are currently in progress.

Interestingly, long-term exposure to oral pimonidazole identified cells that were not hypoxic during the snapshot of hypoxia provided by CCI-103F binding. By analyzing the fraction of tumor cells that were labeled with pimonidazole but did not label with CCI-103F, we were able to identify and quantify cells that were subject to alterations in their hypoxic status (Fig. 3C). That is, we documented tumor cells that were sufficiently hypoxic during the long-term exposure to oral pimonidazole to incorporate that marker, yet were not hypoxic enough to incorporate CCI-103F during the 3 h just before analysis. Importantly, pimonidazole and CCI-103F have been shown to label comparable fractions of hypoxic tumor cells in both solid tumors (11) and spheroids (data not shown). These data suggest that the differences in pimonidazole labeling observed between 6 and 12 h of pimonidazole exposure may be because of transient changes in tumor cell oxygenation, and that a small fraction of cells were subject to even longer-term changes in oxygenation. As the timing of blood flow changes will undoubtedly be quite different in different tumor systems, oral administration of pimonidazole should provide a more convenient way of making such determinations than the multiple injections of the marker used previously to study tumor blood flow dynamics (17). Fig. 3 also reinforces the suggestion that increases in hypoxia measured by long-term pimonidazole exposure were not because of any adverse effects of oral pimonidazole on oxygen supply to the tumors (16). Importantly, the hypoxic fraction of ~500 mg SiHa tumors was ~15% when observed either with CCI-103F or with pimonidazole over a 6-h period, but was close to 30% when pimonidazole was delivered for ≥12 h. Thus, when the measurement of tumor hypoxia was integrated over time through the use of oral pimonidazole, we were able to detect almost twice the number of hypoxic tumor cells as compared with the snapshot of tumor hypoxia given by short-term administration! These data raise the (probably alarming) suggestion that methods of assessing tumor hypoxia over relatively short periods of time might underestimate the therapeutically relevant hypoxic fraction of a tumor.

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