ABSTRACT

Little is known about how and why hypoxia arises in tumors, i.e., whether hypoxia is a chronic process resulting from diffusion limitations or occurs more acutely due to transient changes in blood perfusion. We have investigated the nature of hypoxia in the murine squamous carcinoma SCC VII using a new fluorescence-activated cell-sorting technique which facilitates isolation of viable tumor cells as a function of their distance from the blood supply. The technique utilizes the DNA binding/diffusion properties of the bisbenzamide fluorochrome Hoechst 33342. This compound has a very short half-life from the blood after i.v. injection but remains bound within tumor cells even after disaggregation, redistributing with a half-life greater than 2 h. Cells can thus be sorted on the basis of their staining intensity (proximity to the blood supply), and varying the Hoechst 33342 administration protocol provides a basis for elucidating transient changes in blood flow that result in acute radiobiological hypoxia. Using this technique, we have demonstrated that acute hypoxia results from transient changes in blood perfusion in 500-mg SCC VII tumors. Independent confirmation of the intermittent blood flow has been obtained using histological techniques.

INTRODUCTION

Radioresistant hypoxic cells have been shown to occur in nearly all the animal tumors investigated to date (1). Furthermore, there is now firm evidence that hypoxic cells exist and impair the effectiveness of radiation therapy in some human tumors (2). These hypoxic cells presumably can originate in two ways. The oldest and most accepted model, that of chronic, diffusion-limited hypoxia, is based on the histological observation that the size of the rim of viable cells that surrounds tumor blood vessels invariably exceeds the calculated oxygen diffusion distance (3, 4). This led to the postulate that cells on the edge of the viable cuff bordering necrosis would be hypoxic and therefore resistant to radiation treatment (3). However, over the last decade there has been an increasing body of indirect evidence that another type of hypoxia may exist in tumors, namely acute (transient) hypoxia (5–10). Such hypoxia is thought to occur as a result of temporary cessations of blood flow within the tumor vasculature. Until the present study, there had been no direct evidence that such an intermittent blood flow is a factor in tumor response to irradiation.

We have recently developed a technique which facilitates isolation of tumor cells as a function of their distance from the blood supply (11). This technique utilizes the diffusion/binding properties of the bisbenzamide fluorochrome Hoechst 33342 when it passes through several cell layers. Injection of Hoechst 33342 i.v. into tumor-bearing mice results in a heterogeneous staining pattern with cells close to the blood vessels being brightly fluorescent and those more distant being progressively less fluorescent. This staining pattern, which persists through tissue disaggregation, provides the basis for cell separation using fluorescence-activated cell sorting (12).

After injection i.v., Hoechst 33342 disappears from the blood with a half-life of 110 s (12). As a result, it provides a “picture” of the blood perfusion that existed for the first few min postinjection. This property, together with the fact that tumor cells closest to the blood (and therefore O2) supply during irradiation should be more radiosensitive than those cells more distant, provides a basis for elucidating intermittent changes in blood flow. Initial results using this technique (13) suggested that acute hypoxia was not present in small (200 mg) SCC VII tumors but did occur in larger (>500 mg) tumors, based on data obtained from single tumors of various sizes (175 to 1000 mg). Because of the potential importance to tumor radiobiology of acute hypoxia, we report here a more comprehensive study designed to establish the nature of hypoxia in 500 (±50)-mg SCC VII tumors, the size typically used in this and other tumor systems for therapy-oriented studies. The study has involved the use of the sorting technology developed in our laboratory and also a novel “double-labeling” histological procedure.

MATERIALS AND METHODS

Hoechst 33342. Hoechst 33342 (purchased from Sigma Chemical Co., St. Louis, MO) was dissolved in sterile PBS (NaCl, 120 mmol/liter; KCl, 2.7 mmol/liter; in phosphate-buffered saline, 10 mmol/liter) at a concentration of 1 mg/ml and injected i.v. at 0.25 ml/25-g mouse (i.e., 10 µg/g). Administration i.v. was carried out in one of two ways: (a) a single bolus injection 20 min before the start of X-rays; or (b) by i.v. infusion, using a Harvard microliter infusion pump (infusion rate, 32 to 42 µl/min), during the period of irradiation.

Mice and Tumor. SCC VII tumors (tumors used by enzymic dissociation) were implanted s.c. over the sacral region of the back. For all implants, 2- to 3-mo-old female C3H/He mice were used. Details of the origin, maintenance, and implantation of the SCC VII tumor have been described elsewhere (12). For the present studies, tumors weighing 500 ± 50 mg were selected.

Irradiation Procedures. Irradiation was carried out without anesthesia in a manner similar to that described previously (14) using 270 kVp X-rays at a dose rate of 1.5 Gy min−1.

Preparation of Tumor Cell Suspensions. Twenty min after the end of irradiation, the animals were sacrificed, and the tumor was excised. For each treatment group, 2 mice, each bearing one tumor, were used. (It should be noted that, because tumor blood flow varies from animal to animal as can injection technique, cells from different tumors are not pooled prior to sorting.) Following excision, the tumors were washed with PBS at 4°C, chopped using cross-cut scalpels, and weighed. The resulting fragments after being washed with PBS were disaggregated by gentle agitation for 30 min with an enzyme cocktail of trypsin (0.2%), DNase (0.05%), and collagenase (0.05%). The resulting cell suspension was filtered through polyester mesh (50-µm pore size) and centrifuged, and the cell pellet was resuspended in medium for sorting. Cell suspensions were routinely counted on a hemocytometer enabling tumor cell yield to be ascertained. The mean cell yield for tumors in this series of experiments was 7.8 × 107/g of tissue (SD 2.6 × 107).

Fluorescence-activated Cell Sorting. Full details of the sorting procedure have been described previously (11, 12). In brief, cells are separated on the basis of their Hoechst 33342 concentration (ratio of fluorescence intensity and peripheral light scatter) using a Becton-Dickinson FACs 440 dual argon laser instrument into ten subpopulations (sort fractions). In addition to the ten sorted fractions, an “all sort” was also collected to measure the average response of the tumor cells to treatment.

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2 The abbreviation used is: PBS, phosphate-buffered saline.
Measurement of Tumor Cell Response. Tumor cell viability was assessed after sorting using the soft agar clonogenic assay described previously (15). Known numbers of tumor cells were plated into soft agar and cultured in a water-saturated atmosphere of 5% O₂, 5% CO₂, and 90% N₂ for 14 days. Tumor colonies of more than 50 cells were counted with the aid of a microscope. For the present series of experiments, the plating efficiency (plating efficiency = number of colonies counted/number of cells plated) from untreated tumors ranged from 0.27 to 0.52. By knowing the plating efficiency for each fraction in control and treated tumors, a surviving fraction as a function of decreasing fluorescence can be obtained.

Histological Experiments. For these experiments, orange fluorescent zinc cadmium sulfide particles (5 to 30 μm) purchased from Mono Research Laboratories, Ltd., Shelburne, Ontario, Canada, were suspended in PBS and injected i.v. at a concentration of 4 mg/g mouse either at the same time or 20 min after Hoechst 33342 (50 μg/g). Within 1 min after injection of the microspheres, the animals were sacrificed, and the tumors were excised. The tumors were then frozen and sectioned on a refrigerated microtome. Fluorescence microscopy was performed using a Zeiss microscope with an epicondenser, neofluor objectives, and 100 W mercury light source. Appropriate excitation and emission frequencies were selected by a dicroic filter combination (356-nm excitation and 420-nm barrier filters).

RESULTS

Injection or infusion of Hoechst 33342 into mice produces patterns of intense staining in well-perfused areas of tumors, with little staining in areas distant from the blood supply (Fig. 1). Mice bearing 500-mg tumors were assigned to one of two experimental groups. In the first group, the mice were infused with Hoechst 33342 (10 μg/g) throughout the period of irradiation. Infusion was carried out using a Harvard infusion pump which was started up immediately before the X-ray irradiation and terminated approximately 1 min before the end of irradiation. Using this protocol, it was expected that the Hoechst 33342 staining would provide a fairly accurate picture of the tumor perfusion during irradiation. Cells brightly stained with Hoechst 33342 were much more radiosensitive (more oxic) than cells dimly stained (Fig. 2). Animals assigned to the second group were given injections of Hoechst 33342 (10 μg/g) 20 min before the start of irradiation. If the blood supply changed in this 20-min period such that vessels which were open during the few min postinjection were closed during irradiation and vice versa, we would not expect to see a dramatic difference in radiosensitivity between the bright and dimly fluorescent cell populations. This is indeed what was observed (Fig. 3). One alternative explanation for this effect is that the Hoechst stain is either cytotoxic to or radiosensitizes the brightest sort fraction in the infusion protocol. Indeed, it can be seen from Fig. 4 that the plating efficiency is lower for the brightest sort fraction in both infusion and injection protocols. However, the decreased plating efficiency is unlikely to be the result of Hoechst toxicity, since the previous in vitro studies have shown that no cytotoxicity is observed with a 2-h incubation with Hoechst 33342 in concentrations of 30 μM (12). This corresponds to a fluorescence intensity of 500. The fluorescence intensity of the cell population in the present study only reaches a maximum of 20 (Fig. 5). In addition, the fact that the most intensely stained fractions in the infusion protocol have almost the same amount of Hoechst stain (as measured by fluorescence intensity) as those in the injection protocol would argue against any cytotoxic or radiosensitizing effect being responsible for the survival differences seen. The decreased plating efficiency in the brightest fraction is probably a result of our sorting criteria, since our sorting is carried out on the basis of the ratio of the Hoechst signal divided by the peripheral light scatter signal. Most normal cells are smaller than the malignant cells and preferentially accumulate the stain, so they will be sorted in the brighter sort fractions. Since the normal cells are non-clonogenic, this results in an apparent decrease in plating efficiency in these sort fractions. Of interest from the results shown in Fig. 2 is that radiation resistance increases gradually with decreasing sort fraction, but never reaches the survival value of 2.5 x 10⁻¹ for cells made fully hypoxic (13). This may reflect the fact that our sorting “resolution” decreases with decreasing fluorescence and thus may not adequately isolate a hypoxic population as small as 10 to 20%.

Independent confirmation for the existence of an intermittent blood flow in this tumor was achieved using two fluorescent agents, Hoechst 33342 and zinc cadmium sulfide particles, injected i.v. into the lateral tail vein of mice bearing 500-mg...
**INTERRITENT BLOOD FLOW IN A MURINE TUMOR**

**Fig. 2.** The response of SCC VII tumor cells to 10 Gy of X-ray radiation as a function of their fluorescence intensity after infusion with Hoechst 33342 (10 µg/g) during the radiation treatment. Fraction 1 is the brightest 10% of the tumor cells, Fraction 2 is the next 10% brightest, etc. Bars, SE from 4 experiments. The arrow indicates the survival of the unsorted cell population (“all sort”).

**Fig. 3.** The response of SCC VII tumor cells to 10 Gy of X-ray radiation as a function of their fluorescence intensity after injection with Hoechst 33342 (10 µg/g) 20 min prior to irradiation. Fraction 1 is the brightest 10% of the tumor cells, Fraction 2 is the next 10% brightest, etc. The brightly fluorescent cells are relatively resistant to radiation, indicating that at least some of these cells were low in oxygen during irradiation. This result indicates that the pattern of tumor blood perfusion can alter in a 20-min period. Bars, SE from 3 experiments. The arrow indicates the survival of the unsorted cell population (“all sort”).

**Table 1** Vessels demarcated with fluorescent zinc cadmium sulfide particles but having no visible Hoechst fluorescence

<table>
<thead>
<tr>
<th>Tumors were 500-mg SCC VII.</th>
<th>Particles and Hoechst 33342 injected simultaneously</th>
<th>1/1500 (0%)</th>
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<tr>
<td>Particles injected 20 min after Hoechst 33342</td>
<td>191/1500 (13%)</td>
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SCC VII tumors. Again, the mice were divided into two experimental groups. In the first group both agents were given injections simultaneously. In the second group the particles were injected 20 min after the Hoechst 33342. One min after injection of the particles, the tumors were excised, embedded, and frozen. Using a cryostat, 5-µm frozen sections were cut, placed on slides, and observed under a fluorescence microscope. Since the fluorescent particles do not exit from the capillaries, they provide a graphic marker of blood vessels in the frozen sections. The underlying basis of the procedure is that, when two agents which can both clearly demarcate blood vessels are
Fig. 6. Fluorescence photomicrographs (total magnification, × 220) of tumor sections obtained from an SCC VII tumor 20 min after i.v. injection of Hoechst 33342 (50 μg/g). One min before tumor excision, fluorescent zinc cadmium sulfide particles (4 mg/g) were injected i.v. Most of the vessels, demarcated with particles, were also stained with Hoechst 33342 (A). However, some of the vessels labeled with particles have no visible Hoechst 33342 staining (B).

Injected simultaneously, all the blood vessels open at the time of injection will be labeled with both agents. When the two agents are injected 20 min apart into a mouse bearing a tumor with intermittent blood flow, we should expect to see some vessels stained with only one of the two agents. This idealized criterion is only applicable if both agents can perfuse all the tumor blood vessels; however, the size of the microspheres used in our present study (5 to 30 μm) may prevent some of them entering the smaller vessels and capillaries. Thus, we have only included in our estimate those vessels which contain microspheres and asked whether these vessels were also labeled with Hoechst 33342. It can clearly be seen from Table 1 that, when both agents were injected simultaneously, all the vessels containing microspheres were also labeled with Hoechst 33342. However, when the injection of microspheres was given 20 min after injection of Hoechst 33342, 191 of 1500 areas containing microspheres had no Hoechst labeling, indicating that these vessels, although closed during the few min after Hoechst 33342 administration, were open 20 min later during injection of the microspheres (Table 1; Fig. 6).

DISCUSSION

These independent methods demonstrate that intermittent blood flow can occur in the tumor vasculature of the murine squamous carcinoma SCC VII. Furthermore, this intermittent blood flow has been shown to result in acute hypoxia (as measured by radioresistance) in areas close to blood vessels. This observation can explain many earlier reports that cells selected from grossly viable tumor regions following radiation are at least as radioresistant as those from areas closer to necrosis, and that such cells can provide the foci for tumor
regrowth after radiation treatment (5–7, 16). To our knowledge, no other method is currently available to monitor rapid local changes of oxygen supply in viable cells. The double-labeling technique developed by Franko (17), which involves injecting \([^{14}\text{C}]\)misonidazole and \([^{3}\text{H}]\)misonidazole and detecting the isotope separately using double emulsion autoradiography, does, however, provide an indication of changes in net oxygenation over longer time periods and may thus complement our studies. Although the present study provides little detailed information regarding the time course of the cessations in blood flow and the number of blood vessels affected, close examination of the results indicates that a minimum of 20% of the surface area of the tumor vasculature is subject to such effects which last for periods of at least several min. This result can be derived as follows.

(a) From the histological studies, it is apparent that some vessels which were demarcated with microspheres had no Hoechst staining. Therefore, these vessels must have been closed for several min after Hoechst 33342 injection (distribution half-life of Hoechst 33342 after i.v. injection, 110 s), but were open when the microspheres were injected.

(b) The radiation survival response of the cells most intensely stained following the Hoechst injection protocol, i.e., those in sort Fraction 1 of Fig. 3, can only be explained by either 20% of these cells being chronically hypoxic for all of the irradiation, or 20% of the cells subject to acute hypoxia at any time during the irradiation. This can be derived from the fact that the average cell survival for SCC VII tumor cells rendered hypoxic during 10 Gy of irradiation is \(2.5 \times 10^{-1}\) (13). The observed survival of 20% of the hypoxic survival level thus suggests that either 20% of the surface area of the tumor vasculature was occluded continuously during the irradiation, or > 20% was intermittently occluded.

The occurrence of acute hypoxia in other tumors and the effects of tumor growth rate, size, and site of implantation on such hypoxia are currently being investigated. It is hoped that such studies will provide information on the generality of the effect and why it occurs.

The existence of acutely, as well as chronically, hypoxic cells within tumors has several implications for some of the treatment strategies designed to overcome hypoxic cells. (a) The use of hyperbaric oxygen and high oxygen content gases during irradiation, designed to be effective in radiosensitizing chronically hypoxic cells (by increasing the \(O_2\) diffusion distance outwards from open blood vessels), would be expected to have little or no effect on acutely hypoxic cells. (b) Drugs selectively toxic to hypoxic cells but requiring contact times of hours may not be as toxic to acutely hypoxic cells alternating between aerobic and hypoxic states (though, interestingly, sufficiently active agents would then be predicted to show toxicity in both well- and poorly vascularized regions of the tumor). However, radiosensitizing drugs, such as the nitroimidazoles, which are small freely diffusible compounds and as a result can be equally distributed throughout the tumor areas, should be as effective in sensitizing both acutely and chronically hypoxic cells.

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