Detection of an Intrinsic Marker in Hypoxic Cells

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INTRODUCTION

Despite the widely held belief that hypoxic cells limit the efficacy of radiotherapy, treatment strategies specifically designed to overcome the problem of tumor hypoxia have offered no sweeping improvement. Current thinking is that hypoxic cells may present a problem only in the treatment of certain tumors. It would therefore be therapeutically advantageous to identify these tumors and have a means of estimating their hypoxic fractions (1, 2). Labeled compounds which preferentially bind to hypoxic cells are currently being developed for this purpose. Radiolabeled misonidazole (3) has been the most widely utilized of these compounds to date, and some patient studies have been conducted using [3H]misonidazole (4). Other nitroheterocycles are also being employed using radiolabels, fluorescence, and magnetic resonance spectroscopy (2, 5-7). Although extremely useful, such drugs do require delivery to and uptake by the tumor tissue. The need for the injection of a marker can be circumvented if an intrinsic marker of hypoxia can be detected.

We have detected intrinsic macromolecular changes in chronically hypoxic cells which act as a “fingerprint” of hypoxia. Using the sandwich system, it was noted that interior cells which had been chronically hypoxic and nutrient deprived could chemically reduce autoradiographic emulsion, resulting in the production of grains. These silver grains are of the same nature as seen in standard autoradiographic studies, but here they are produced by chemical reduction of the emulsion rather than radioisotope exposure. Chemographs are known sources of artifacts in radiography experiments (8). Procedures for eliminating such artifacts include putting a thin impermeable membrane or a layer of evaporated carbon between emulsion and sample (8-10). Usually the specific chemical reducing the film is unknown (11-13) and of no particular interest to the experimenter. But, in our case, it appeared that the ability to produce chemographic grains could be correlated with the degree of cellular hypoxia and this could be done in a quantifiable way.

The sandwich system, used in these studies, is an in vitro, multicell tumor analogue. Sandwiches, like spheroids, exhibit central regions of dead cells and cell debris surrounded by viable cell borders (14). Over the width of the viable border there exist spatial gradients of nutrients and oxygen. The oxygen gradient one achieves in the sandwich mimics an oxygen gradient typically expected in poorly vascularized tumors. Within sandwiches, the spatial profile of chemographic grains per cell as a function of distance into the sandwich was observed to be quite similar to the profile obtained (15) for radiographic grains from [3H]misonidazole labeling. This suggests that, at least in vitro, the same low oxygen tensions which give rise to substantial MISO binding also promote biochemical changes which are detectable by the chemical reduction of emulsion.

MATERIALS AND METHODS

Studies were conducted using sandwiches of the following cell lines; 9L rat gliosarcoma, V79 Chinese hamster fibroblasts, Chinese hamster ovary, EMT6 mouse mammary carcinoma, and MCF-7 human breast cancer. The results were qualitatively similar regardless of cell line. Monolayer and sandwich cultures of the V79, 9L, and CHO lines were grown in Eagle’s minimal essential medium with Earle’s salts (GIBCO), supplemented with glutamine, 11% newborn calf serum (GIBCO), and 4% fetal calf serum (Irvine Scientific); bicarbonate buffer was added. For the EMT6 line Waymouth’s media plus 10% fetal calf serum and for the MCF-7 line RPMI plus 10% fetal calf serum were used.

Sandwich Culture. In the sandwich system, cells grow in a narrow gap between two glass microscope slides separated by spacers of 150 μm. The cells are grown in a monolayer attached to the bottom slide and medium completely fills the gap between the slides. Sandwiches are assembled and held in place in a petri dish which acts as the medium reservoir. In order for the inner cells of the monolayer to be continuously supplied with nutrients, nutrients must diffuse from the medium reservoir through the narrow gap between the slides (Fig. 1). The competition between diffusion and cellular consumption of nutrients and oxygen sets up nutrient and oxygen gradients. Cell heterogeneity within the monolayer develops as a result of these gradients. In time, cells in the center of the slide die from nutrient and oxygen shortages and a “necrotic center” appears. Cells are initially seeded on the bottom slide at a density of 0.5 x 10^6 per slide and they are covered with a top slide 24 h after seeding. For more details on the sandwich method see (14).

Chemographic Studies. In these chemograph studies, the sandwiches were left undisturbed in the incubator for 48-72 h following covering with a top slide. At times ranging from 48 to 72 h after covering, the top slide was removed and the cells fixed in situ on the bottom slide. By the time of fixation the sandwich culture is well developed: it has a viable cell border, within which gradients of local cell environment and cell state have already been established, and has a necrotic center. For fixation, sandwich slides (and control monolayers) were rinsed with PBS, fixed in 3:1 EtOH:acetic acid, followed by a 70% EtOH rinse, and then air dried. It is important to note that reoxygenation of all cells occurs within a matter of seconds after removal of the lid; therefore
cells are reoxygenated prior to fixation. The details of the dipping and development of the emulsion seem crucial to the detection of chemographs. Slides were dipped in either Kodak NTB2 or NTB3 emulsion; the slides were boxed while the emulsion was still wet and developed after 3–14 days. The emulsion being wet for an extended period of time may facilitate the chemical exposure of the film. To further enhance the chemical exposure of the film, the slides were developed at a higher temperature, 18°C, and for a longer period, 7 min, than used for development of radiographs. Following development the slides were stained with hematoxylin.

**[3H]Misonidazole Studies.** To augment these studies replicate V79 sandwiches were labeled with [3H]misonidazole (generously provided by Dr. J. A. Raleigh) to identify and locate hypoxic regions for comparison with regions of chemography. Sandwich and monolayer cultures were labeled at a concentration of 65 µM with a labeling time of 24 h. The specific activity was 584 µCi/mg. Sandwich cultures were labeled 50 h after covering and were labeled with the lids in place. Warm medium containing the MISO was added to the cultures and they were incubated (37°C) for the labeling period. Following labeling, the radioactive medium was drawn off and the slides were rinsed twice with medium containing no MISO. For sandwich cultures, the top slide was removed during this rinsing period to allow maximum efficiency in rinsing out the unbound MISO. Following rinsing, medium without MISO was again added and the slides were incubated at 37°C for an additional 15-min period. The slides were then rinsed three times in PBS, fixed in 3:1 EtOH:acetic acid, rinsed in 70% EtOH, air dried, and dipped in Kodak NTB2 emulsion. In order to secure that chemographic grains would not contaminate our MISO counts the slides were boxed and developed in a way inconsistent with producing chemography; the slides were dried thoroughly prior to boxing; and they were developed at an insufficient temperature and for an insufficient period of time (16°C for 3 min). Following 20 days of exposure the slides were developed and stained with hematoxylin. Following development the slides were carefully examined visually for any evidence of chemographic grains, which have a less uniform size distribution. Conversely, radiographs from [3H]MISO could not have contaminated the chemographic counts since no MISO label was given in the chemograph experiments.

For purposes of quantification of the chemographs or the radiographs, slides were scored for grains per cell as a function of distance into the sandwich. The slides were also scored for labeling index (fraction of labeled cells in the population) as a function of distance into the sandwich.

**RESULTS**

When cells are grown in sandwich culture one achieves, on a single slide, a monolayer population subject to a smooth distribution of growth conditions. The conditions range from plentiful oxygen and nutrients to oxygen and nutrient deprivation severe enough to cause cell death. Comparing cells in different regions of the slide permits one to observe changes in cell state evoked by the various ambient growth conditions. This enabled us to observe that hypoxic, nutrient-deprived sandwich cells had the potential to chemically reduce autoradiographic emulsion. That is, cells in the interior, hypoxic, nutrient-deprived regions of sandwiches (Fig. 1) produced chemographic grains in emulsion while those near the oxygen and nutrient source, in the exterior regions of the sandwich, did not. Fig. 2 shows cells from the interior border region, adjacent to the necrotic center, of a 9L sandwich. A large number of chemographic grains are seen in the emulsion over these cells. This sandwich culture was not labeled with any radioisotope and reduction of the emulsion is due to chemicals in the cells.

Viewing the sandwich viable border as a whole, cells in the inner region of the viable sandwich border, had been chronically hypoxic and nutrient deprived. No radiolabeled compound was used in the experiment shown here.

**Fig. 1. Cross-section of a sandwich.** Cells are grown in monolayer on the bottom slide. When the top slide is subsequently put in place, a balance between cell consumption rates and diffusion rates determines the gradients of oxygen, nutrients and metabolites in the x direction. x is the distance into the sandwich, i.e., the distance from the source of nutrients and oxygen.

**Fig. 2. Chemographic grains in the emulsion over 9L cells.** These cells, from the inner region of the viable sandwich border, had been chronically hypoxic and nutrient deprived. No radiolabeled compound was used in the experiment shown here.
Fig. 3. A plot of the average number of chemographic grains per cell, for V79 sandwiches, as a function of distance into the sandwich. The necrotic centers occurred at ≈2 mm.

Fig. 4. Histogram showing the distribution of chemographic grains per cell at 1.7 mm into a V79 sandwich.

Table 1. Statistics on chemographic grains per cell at 0.3 mm into sandwiches.

<table>
<thead>
<tr>
<th>Number of grains</th>
<th>Number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No detectable grains</td>
<td>29,650</td>
</tr>
<tr>
<td>5–9</td>
<td>4</td>
</tr>
<tr>
<td>10–19</td>
<td>55</td>
</tr>
<tr>
<td>20–29</td>
<td>43</td>
</tr>
<tr>
<td>30–39</td>
<td>10</td>
</tr>
<tr>
<td>40–49</td>
<td>8</td>
</tr>
<tr>
<td>50–59</td>
<td>10</td>
</tr>
<tr>
<td>60–79</td>
<td>5</td>
</tr>
<tr>
<td>80–100</td>
<td>2</td>
</tr>
</tbody>
</table>

The fraction of detectably labeled cells is ≈4 × 10⁻³.

Computed from the data in Fig. 4 is larger than can be accounted for by Poisson statistics alone.

Although the vast majority of the cells in the outermost regions, next to the nutrient source, showed no detectable chemographic grains, a small fraction of these cells did show a quite significant amount. This bimodal response at 0.3 mm into the sandwich, an essentially unlabeled region, is documented in Table 1. Out of ≈30,000 cells scored, 137 cells showed detectable grains. Of these 137 cells, the highest number of grains per cell detected was 100 and the lowest was 6. There should be no difference in the response of cells in the outermost strips of sandwiches and in control monolayers. We therefore examined control monolayers for the presence of a low frequency of individual cells showing chemographic grains when covered with emulsion. For V79 control monolayers ≈2 × 10⁻³ showed detectable grains, essentially the same fraction as seen for the outer regions (0.0–0.3 mm) of covered sandwiches.

A sandwich viable border contains a mixture of cells with and without chemographic grains. For the sandwiches of Fig. 3 the fraction of cells with chemographs in each 0.2-mm band was counted as the labeling index. A plot of the labeling index as a function of distance into the sandwich is shown in Fig. 5. As was the case for the number of grains per cell, the labeling index increases with distance into the sandwich. The fraction of labeled cells increases from ≈4 × 10⁻³ near the nutrient source to 100% in regions adjacent to the necrotic center. In the region from 0.4 mm to 1.1 mm the rise of the labeling index is approximately exponential.

Note, a sandwich profile of chemographic grains (e.g., Fig. 3) can be compared with sandwich profiles for other endpoints and other labels such as DNA content, [³H]thymidine labeling, [³H]misonidazole labeling, etc. In Fig. 6 a profile of [³H]MISO labeling for V79 sandwiches is shown superimposed on that of chemographs. [³H]Misonidazole is a radiolabeled nitroimidazole which preferentially binds to hypoxic cells and has been used as a label for hypoxia in tumors and tumor models (3, 4, 6).
A large number of cells from different replicate V79 sandwiches were pooled to obtain these averages. The average number of grains, for both chemographs and radiographs, are shown as a percentage of the observed maximum, as noted on the right axis. The curves have very similar shapes, demonstrating that it is cells in the same region of the viable border (i.e., in the same microenvironment) which show both the chemo-
graphic grains and the [3H]MISO labeling. In fact, the chemographic response in the [3H]MISO labeling was not made.

A chemographic "labeling index" was also measured for the sandwiches, since a bimodal cellular response (cells with no grains and cells with an appreciable number of grains in the same microenvironment) was noted. This is a quite different response from that seen with [3H]MISO binding, where all cells within a given microenvironment show similar degrees of labeling, with the observed variations in grains per cell apparently attributable to a combination of Poisson statistics and variations in cell size. The labeling index profile indicated 100% of the interior border cells showed chemographic grains; seemingly all cells have responded to the adverse environmental conditions. But it was surprising to note that ≅0.5% of the cells in the outermost 0.2-mm strip also showed chemographs, since cells in the outermost strip of a sandwich experience a local environment essentially like that of an oxygenated monolayer (20% O2). This suggested that a similar response in oxygenated monolayers was to be expected. Monolayer cultures were examined and indeed ≅0.5% of the cells showed chemographic grains over their cytoplasm. This presumably indicates that factors other than oxygen concentration can trigger the response, although variation in intrinsic oxygen susceptibility is still a possibility. The anomalously labeled cells occur either as single labeled cells amidst neighboring unlabeled cells or as small clusters of labeled neighboring cells in unlabeled regions (suggestive of genetic factors or division following manufacture of reducing substance). In any case, the sandwich chemographic labeling profiles (along with N2 monolayer data and human tumor studies to be published) indicate that O2 deprivation dramatically amplifies the response.

Reducing agents, e.g., glutathione, cysteine, uric acid, and hydroquinone are capable of producing a latent image in silver halide crystals by direct chemical action (8). This kind of chemical action is far more likely when using specimens which have not been paraffin embedded, and possibly explains why chemographs have not been seen in spheroid sections. The appearance of chemography is also very sensitive to conditions of exposure and development of the emulsion. This sensitivity enhances the difficulties of quantification with this type of indicator.

The amount of literature on chemography is rather scanty. This is presumably because, as mentioned, chemography is often regarded as an artifact to be avoided (9, 10) rather than an indicator of cellular state and it is technically difficult to get reproducibility. Some of the difficulty can be ascribed to the lack of specificity of the autoradiographic emulsion for this application. Mulvaney (18) did investigate a source of chemography at the electron microscope level and found that particular lysosome-like structures of rabbit olfactory epithelium give...
rise to chemographs while others do not. He found that the lysosome-like inclusion bodies which produce chemography closely resemble "pigment granules" which increase with age of the animal. A paper dealing with cells in culture rather than tissues is that of Silk et al. (19). They presented a method for "high resolution" intracellular autoradiography for the electron microscope. The experimental results indicate cytoplasmic grains adjacent to the nucleus. \([\text{H}]\)Thymidine was used in these studies and these grains were interpreted as thymidine uptake in the cytoplasm, but we believe that it is far more likely that they were seeing chemography resolvable on the electron microscope level.

The substance responsible for the chemography seen in the oxygen-deprived cells of our study is unknown at present. The fact that it is not washed out of the cells during the fixation process suggests that it is bound. Preliminary studies indicate it is membrane associated. The timing for the decay of the effect (discussed below) suggests it could be a protein. It is well known that certain stress proteins are manufactured in response to dire environmental conditions such as hypoxia (20, 21). It is also known that under certain conditions the Golgi apparatus has the capacity to reduce silver halide. In fact, it was by way of his chromo-argentie impregnation technique that Golgi first visualized the organelle through the light microscope (22). With further investigations these clues could presumably come together and enable us to identify the mechanisms involved.

In order to be limiting to therapy, the hypoxic cells in the interior of tumors must be viable. It is necessary to determine if cells showing chemography are viable following reoxygenation. \([\text{H}]\)Thymidine labeling studies (23) show that \(\approx 80\%\) of the cells in the hypoxic regions of 9L and V79 sandwiches resume cycling after reoxygenation. Cells in the photos, Figs. 2 and 7, do not show characteristics that would categorize them as nonviable. The sandwich cells that express chemography were explicitly shown to be still viable. If slides were returned to the incubator after reoxygenation then, within 24 h, the previously hypoxic cells resumed proliferation and also gradually lost the ability to reduce emulsion.

In conjunction with these studies, and to be reported on elsewhere, hypoxic monolayers (9L, V79, EM76, MCF-7), cross-sections of human cervix tumors were tested for chemography. All showed subcellular chemography, with a similar cytoplasmic localization, which we believe stems from a common source. The results support the conclusion that hypoxia promotes chemical changes which result in the potential to reduce chemographic emission. It should be remarked that in the case of tumor sections, the grains, found in localized regions of the specimen, were not always discrete and could not be counted as was done in this study with sandwich cultures.

An intrinsic marker offers the possibility that fresh tissue may be processed in such a way that regions of hypoxia are detectable without the worry of label delivery to the tumor site. In this way the extent of hypoxia in particular tumors can be evaluated, making for enlightened radiation and chemotherapy treatment protocols. These chemographs are not necessarily the marker of choice but, at the very least, give indication that there are appreciable detectable changes in the cell's biology after prolonged hypoxia.

Cellular hypoxia is also of interest in a number of other medical applications (ischemia, brain hypoxia, reperfusion injury, oxidative stress, renal hyperperfusion, prenatal hypoxia, etc.) as well as in fundamental biological studies, e.g., of cellular necrosis (24). Intrinsic hypoxia markers could be valuable in such areas also.

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REFERENCES

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