

Hypoxia and Perfusion Measurements in Human Tumors

Initial Experience with Pimonidazole and IUdR

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Acta Oncologica Vol. 40, No. 8, pp. 924–928, 2001

We describe our preliminary studies on the development of methods to measure hypoxia in standard paraffin sections of human tumors. Three parameters were investigated. First, image analysis of tumor vascularity yielded the parameter diffusion limited fraction (DLF), which is the amount of tumor tissue greater than a fixed distance from the nearest blood vessel. Secondly, the amount of tumor tissue stained with antibodies against bound reduced products of the bioreductive marker pimonidazole was assessed. Finally, the fraction of blood vessels showing no surrounding tumor tissue labeled with IUdR, a cell kinetic marker, was measured. DLF and pimonidazole monitor primarily chronic hypoxia, while it is hypothesized that the IUdR-negative fraction monitors acute hypoxia. Feasibility was demonstrated in a series of 10 esophageal and 10 rectal tumors (no drug administration), 10 cervix tumors (pimonidazole) and 14 head and neck tumors (pimonidazole and IUdR). Significant differences between tumors were found for all parameters. DLF correlated significantly with the pimonidazole fraction when all images of all tumors were included, although mean values per tumor showed no correlation. The IUdR-negative fraction did not correlate with either of the other two parameters. We conclude that it is feasible to measure hypoxia-related, and possibly perfusion-related, parameters on paraffin sections for predictive purposes, although each method needs further validation. Each parameter will be correlated with outcome in a larger study on head and neck tumors treated with surgery with or without postoperative radiotherapy.

Received 5 October 2001

Accepted 15 October 2001

The importance of tumor hypoxia as a negative prognostic factor continues to be confirmed and strengthened by each new publication showing a significant correlation between microelectrode measurements of tumor oxygenation and outcome. This applies to all of the three major modalities of cancer treatment, i.e. surgery, radiotherapy and chemotherapy (1). Continued investigation into the causes, consequences and treatment of hypoxia is therefore of great interest and importance, not the least in order to ensure the most efficient application of treatments such as ARCON and Tirapazamine, which are designed to attack or exploit hypoxia (2–4).

Almost all the microelectrode measurements made in the past few years using the Eppendorf histograph are restricted to accessible tumors only. In addition, there are aspects of tumor biology related to hypoxia that are not readily amenable to study using oxygen electrodes, partic-

ularly the relative importance of chronic and acute forms of hypoxia, and the importance of intermediate hypoxia. The relatively large collecting volume of the present clinically used electrodes precludes drawing any conclusions on these aspects. In the present study, we investigated a vascular-based parameter—diffusion limited fraction (DLF)—and a bioreductive marker (pimonidazole; (5)) to estimate hypoxia, and the use of a cell kinetic marker (IUdR) to indicate perfusion in individual vessels (6). This paper presents a preliminary report on our findings to date.

MATERIALS AND METHODS

Parameters

We studied three main parameters which relate to hypoxia and perfusion (see Fig. 1): 1) the diffusion limited fraction (DLF): a parameter for estimating chronic, diffusion lim-

ited hypoxia from image analysis of blood vessels. This comprises the fraction to tumor tissue greater than a fixed distance (usually set to 120 μ) from the nearest vessel; 2) the pimonidazole fraction: a fraction of tumor tissue stained with antibodies to bound, reduced products of this bioreductive marker drug; and 3) the fraction of blood vessels around which no IUdR labeling of tumor cells is seen. The hypothesis is that lack of labeling is caused largely by lack of perfusion, preventing delivery of the marker (6).

DLF and pimonidazole are measures primarily of chronic hypoxia, while the IUdR negative fraction is assumed to be primarily a marker of acute hypoxia.

Patient material

For the DLF studies, fresh biopsy samples from 10 randomly chosen patients with squamous cell carcinoma of the esophagus and 9 with adenocarcinoma of the rectum were studied. Tumor biopsies were frozen in liquid nitrogen and stored at -80°C until frozen sections were cut.

For the cervix tumors, 0.5 g/m² pimonidazole hydrochloride was infused intravenously over 20 min and tumor biopsy material was taken 24 h later, fixed in neutral buffered formalin prior to paraffin embedding and sectioning, as described (7).

For the pimonidazole and IUdR studies, resection material from 20 consecutive unselected patients with advanced head and neck tumors and planned for surgery with or without postoperative radiation therapy was investigated. These patients received 50 mg/m² IUdR as an i.v. bolus, and then 15–20 min later 0.5 g/m² pimonidazole hydrochloride infused intravenously for 20 min. Tumor material was taken 12–16 h later during surgery and processed for paraffin sectioning. Of the 20 patients, 6 could not be analyzed owing to a too short pimonidazole-operation interval (2 patients), decalcification of

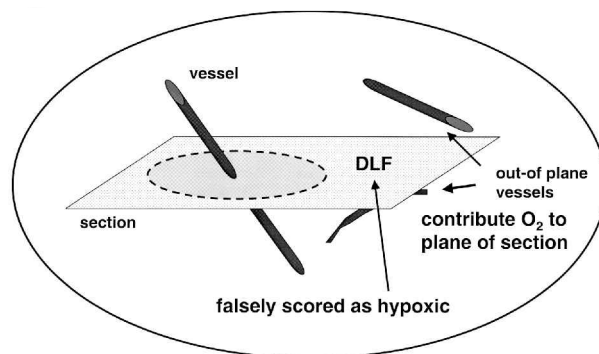


Fig. 2. Schematic showing how the DLF (diffusion limited fraction) will overestimate the fraction of chronically hypoxic cells by not taking into account out-of-plane blood vessels. This may or may not influence the ranking of tumors.

specimen, destroying epitopes (3 patients) and too little material (1 patient).

Immunohistochemistry

To stain blood vessels, a mouse anti-human CD31 was used, followed by a biotinylated goat anti-mouse second antibody and a tyramide amplification step. For pimonidazole, a biotinylated anti-pimonidazole antibody was used, followed by an avidin-biotin-peroxidase complex. For double staining, peroxidase activity was detected with DAB/Ni (black) for CD31 and DAB (brown) for pimonidazole. Slides were lightly counterstained with hematoxylin. Staining on esophagus and rectal tumors was done on frozen sections, and on paraffin sections for cervix and head and neck tumors.

Image analysis

Images were made at a magnification of $5\times$ with an inverted microscope mounted to a black-and-white CCD camera. DLF analyses were carried using an application written in SCIL-Image (7). Briefly, non-tissue areas were excluded, a pathologist delineated areas of tumor tissue, staining artifacts were then removed and the proportion of tumor tissue area greater than a fixed distance from the nearest stained blood vessel calculated. A region round the edge of each image equal to the chosen diffusion distance was excluded from the calculations, so that these edge regions would not be falsely regarded as 'hypoxic' if there was a blood vessel just out of the image. This does not correct for out-of-plane vessels, which however, could have contributed to oxygenation in the section analyzed (Fig. 2). Pimonidazole-positive areas within the selected tumor areas were measured by thresholding on the pimonidazole stain. The percentage of vessels having no surrounding IUdR label was scored under the microscope on sections double stained for vessels (CD31) and IUdR.

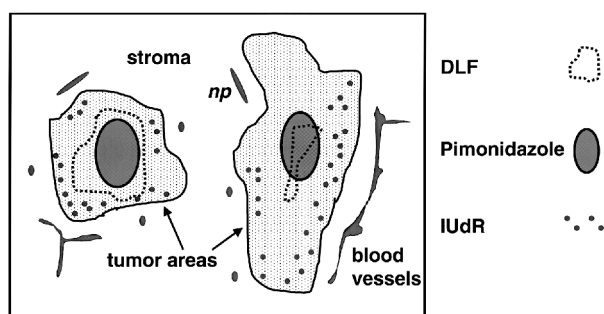


Fig. 1. Schematic illustration of the three parameters for estimating hypoxia and perfusion in paraffin sections of human tumors. Patients are given the bioreductive drug pimonidazole and the cell kinetic marker IUdR 12–16 h before surgical removal of the tumor. DLF: diffusion limited fraction; np: non-perfused vessel, having no IUdR labeled tumor cells in the vicinity.

RESULTS

Hypoxia estimates based on vasculature

The DLF parameter was first tested on a series of 10 esophageal tumors. As a positive control, the CD31 antibody was omitted, and showed, as expected, DLFs greater than 95%, indicating that in the absence of detectable blood vessels, most of the section was seen as hypoxic. Normal striated muscle tissue was stained with CD31 as a negative control, showing DLF values of 2–7% at 120 μ . DLF values in esophageal tumors ranged from 2% to 49% (means of 10–15 images per tumor). On a series of 15 different images from an esophageal tumor, intra- and interobserver variability was found to be low (correlation coefficients of 0.99 and 0.93, respectively), confirming the reproducibility of the method.

Similar DLF analyses were carried out on a series of 9 rectal tumors and 10 cervix tumors. In all tumors, intratumoral variability was considerable, indicating heterogeneity of tumor vascularization. Despite this variability, significant differences between tumors were observed for all sites. Mean DLFs and standard deviations at 120 μ were 17% \pm 14% (esophagus), 29% \pm 19% (rectum), and 34% \pm 15% (cervix). The intertumor variations were significantly larger than the intratumor variations, a necessary criterion for a predictor. In a second series of 14 head and neck tumors, the mean DLF was 28%, again with significant differences between tumors. The results for head and neck tumors will be reported in detail elsewhere.

Hypoxia estimates based on a bioreductive marker

Pimonidazole fractions were first measured in a series of 10 cervix tumors (University of North Carolina), and subsequently in a series of 14 head and neck tumors (The Netherlands Cancer Institute, Amsterdam and University Hospital Gasthuisberg, Leuven, Belgium). Multiple images for each tumor were analyzed. In most sections pimonidazole staining was seen mostly at a distance from blood vessels, consistent with diffusion limited hypoxia. In the cervix carcinomas, 1–6 biopsies were studied per tumor. A wide variation in the pimonidazole stained fraction was found between images in one tumor and between means for the different tumors. Despite intratumoral variation, there were significant differences between tumors ($p = 0.02$; ANOVA). A further series of 14 patients with head and neck cancer was then studied. As with cervix tumors, large intra- and intertumor variations were seen, and an analysis of variance showed a highly significant difference between tumors.

We also observed pimonidazole staining which appears to be associated with areas of keratinization occurring in some squamous cell carcinomas. These regions often have sharp demarcations, as opposed to the more gradual increase in staining with distance from blood vessels usually seen in a tumor cord. This suggests that some staining

might be due to non-specific binding associated with keratinization rather than hypoxia. Similar staining was seen in some areas of normal mucosa. It is possible, however, that the differentiated cells are metabolically active and capable of binding hypoxic markers. This possibility has been suggested previously in a comparison of involucrin and pimonidazole immunostaining in squamous cell carcinomas (8). Nevertheless, the possibility that pimonidazole binding to regions of keratinization represents artifactual staining, possibly related to the use of a biotinylated anti-pimonidazole antibody, is a subject of an ongoing investigation.

Perfusion estimates based on IUdR

We hypothesized that one of the causes of heterogeneity in tumor cell labeling seen with IUdR, a marker for active DNA synthesis, was heterogeneity in the supply of the marker caused by perfusion variations between individual blood vessels. This was supported by our preclinical data on mouse tumor models (6). We therefore scored the individual blood vessels on a five-point scale according to surrounding IUdR labeling of tumor cells. Of particular interest were vessels showing no labeling (see vessel labeled 'np' in the schematic in Fig. 1). In the first two head and neck cancer patients, who received both pimonidazole and IUdR the night before surgery, there was a wide distribution of labeling around vessels, with the negative fraction (score = 0) being 1% and 8.6% (see Fig. 3 for results of the second patient). In this tumor, MIB1 labeling was seen around almost all blood vessels. This implies that the lack of IUdR labeling was not due to lack of proliferating cells, and thus supports the hypothesis that labeling heterogeneity is due to perfusion variations.

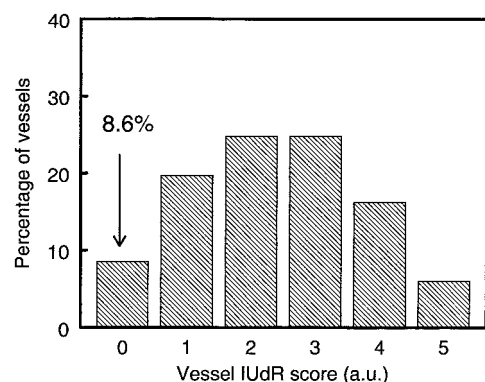


Fig. 3. Heterogeneity of IUdR labeling of tumor cells around individual blood vessels in a human squamous carcinoma of the head and neck. Labeling was scored on an arbitrary scale from 0 (no label) to 5 (maximum labeling). A significant fraction of vessels had no surrounding label, leading to the hypothesis that these vessels were non-perfused at the time of IUdR administration.

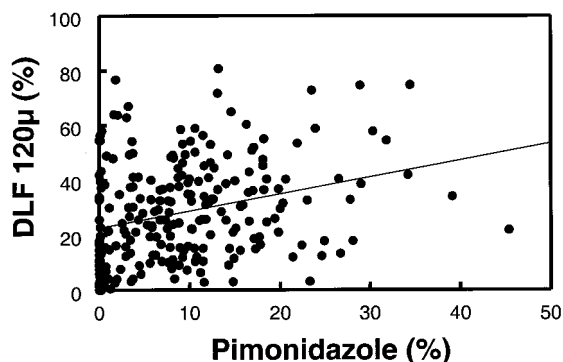


Fig. 4. Correlation of diffusion limited fraction (DLF; tumor fraction greater than $120\ \mu$ from the nearest blood vessel) with the pimionidazole-stained fraction in head and neck tumors. Each point represents one image; a total of 237 images. Despite the large scatter, as evidenced by the low correlation coefficient ($r = 0.29$), the correlation was highly significant ($p < 10^{-5}$).

Correlations between parameters

DLF and pimionidazole fractions in individual images were compared in both the cervix series and the head and neck series. Whether looking at individual biopsies or whole tumors, there was invariably a trend toward higher DLF values with higher pimionidazole fractions. In the cervix tumors, significant correlations were seen in 3 out of 5 tumors in which more than 2 biopsies were analyzed (7-46 images per tumor). In the head and neck tumors, there was a trend toward higher DLFs with higher pimionidazole fractions in the majority of tumors. When all images were analyzed together, there was a highly significant correlation, although a large scatter (Fig. 4). A significant correlation with large scatter was also seen for cervix tumors ($p < 10^{-5}$, $n = 123$). Of note is that the DLF values were all considerably higher than the pimionidazole fractions (see below).

DISCUSSION

These studies show the feasibility of measuring multiple parameters related to hypoxia in human tumors using standard immunohistochemical detection on paraffin or frozen material combined with image analysis. The parameters chosen for study here are not the only ones available. There is an increasing list of genes that are upregulated under hypoxic conditions, the archetype being HIF-1 α (hypoxia inducible factor). These are exciting considerable current interest as potential endogenous markers of tumor hypoxia, and have the advantage that no drug need be administered to the patient. HIF-1 α is a transcription factor leading to expression of several other genes, including VEGF (vascular endothelial growth factor), glucose transporters, and carbonic anhydrases (9, 10). These already appear to show promise as prognostic markers (10, 11). We will report on our results with endogenous hypoxic markers separately.

The parameters described here were chosen so that both chronic hypoxia (DLF, pimionidazole) and acute hypoxia (IUdR) could be estimated in the same tumors. DLF has the advantage of requiring no administered drug, it can be carried out on archival material, and previous studies have shown a relationship between vasculature and outcome (12-16). DLF has several disadvantages, however. It is indirect, and assumes a constant oxygen tension in all vessels, the latter being clearly not the case (17). However, in order to rank tumors according to chronic hypoxia, what is required is that variations in oxygenation within vessels are similar between tumors. This is a less stringent condition and not implausible, but needs further study. DLF also suffers from the fact that out-of-plane vessels, which will contribute oxygen to in-plane tumor tissue (Fig. 2), cannot be taken into account. This will result in overestimation of hypoxia, consistent with the findings that DLF values are always larger than pimionidazole fractions. Again, if this factor is approximately constant between tumors, it may not disturb ranking. There is also the geometric consideration that the distance from vessel to hypoxia will be influenced by the cut angle of the section (Fig. 5). Despite its drawbacks, however, it appears to correlate significantly but weakly with the pimionidazole fraction. The usefulness of DLF will only become apparent when correlations with outcome are made.

Pimionidazole stains cells primarily at a distance from the blood vessel, consistent with chronic hypoxia. It has also been reported to co-localize to a large extent with the differentiation marker involucrin, and it has been suggested that hypoxia might affect oxygen-regulated gene expression in squamous cell carcinomas (8). We have also found pimionidazole staining close to regions of keratinization in some tumors, but whether these cells are hypoxic or represent non-hypoxic immunostaining is not yet clear. This deserves attention and clarification if pimionidazole and other bioreductives are to be used reliably as quantita-

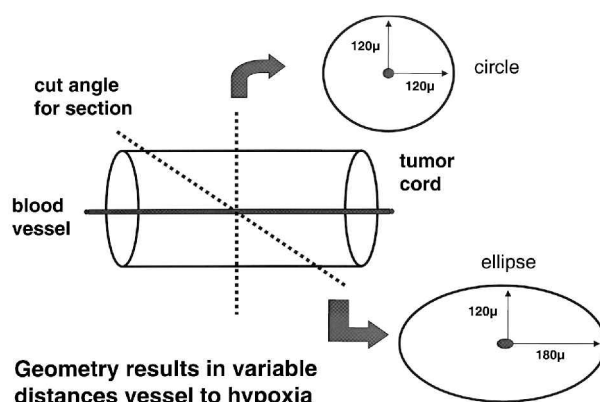


Fig. 5. Schematic illustration of geometric factors influencing the perceived distance from blood vessel to hypoxia or necrosis on a histological section of a tumor.

tive hypoxic markers. The staining patterns in the majority of tumors studied here, however, suggest that it indeed monitors diffusion limited hypoxia.

Measurement of acute, or perfusion limited, tumor hypoxia is difficult in man, especially at the level of individual vessels. No human equivalent perfusion markers, such as the Hoechst dyes used in animal models (18), are available. We have therefore attempted to exploit the observed heterogeneity of labeling by the thymidine analog iododeoxyuridine, a drug often used in the clinic, as indicating microregional variations in perfusion. Although our preclinical studies support this hypothesis, further validation is needed. The combination with endogenous proliferation markers such as MIB1 should help in this regard. Lack of IUdR labeling could be caused by absence of proliferating cells. However, if MIB1 labeling is seen in IUdR-negative regions, the lack of IUdR labeling would not be due to lack of proliferating cells but more likely due to lack of perfusion. This appears to be the case in the head and neck tumors studied to date, but this interpretation depends on the extent to which endogenous markers persist following hypoxia induction and cell cycle cessation. More work in this area is needed.

Significant correlations between DLF and pimonidazole might be expected, since both primarily monitor diffusion limited hypoxia. This was indeed the case when all images were analyzed together, and for several tumors when analyzed individually. We are now investigating whether the lack of correlation in some tumors is due to problems with pimonidazole (e.g. highly differentiated-related staining) or to problems with DLF (e.g. missing some in-plane blood vessels). We are also currently embarking on a larger trial including 150 patients with head and neck tumors in which we will correlate these parameters, and endogenous markers for hypoxia, with outcome.

ACKNOWLEDGEMENTS

We are indebted to Julie Denekamp, a good friend and colleague, who provided enormous stimulation, encouragement and many interesting ideas on hypoxia over many years (19). This study was funded partly by an NIH grant 1R21CA80146-01A1 and partly by a Dutch Cancer Society grant NKI 2000-2202.

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