

# Measurement of hypoxia using invasive oxygen-sensitive electrode, pimonidazole binding and $^{18}\text{F}$ -FDG uptake in anaemic or erythropoietin-treated mice bearing human glioma xenografts

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**Abstract.** Relationship between haemoglobin levels and tumour oxygenation has been already reported. The purpose of this work was to compare in human malignant glioma-bearing mice the sensitivity of two well established techniques of tumour hypoxia assessment, especially their ability to detect expected weak variations of tumour oxygenation status associated to haemoglobin level modifications. The relationship between tumour hypoxia and glucose metabolism was also investigated. Experiments were performed on a human malignant glioma (GBM Nan1) xenografted into *nude* mice. Twenty-four hours after tumour implantation, animals were randomized into three groups: 'Anaemia' for mice subjected to repeated blood samplings, 'Control', and 'rHuEPO' for mice receiving recombinant human erythropoietin. Once the tumours reached a volume of  $300 \pm 100 \text{ mm}^3$ , tumour hypoxia was assessed both using the  $\text{pO}_2$ -Histograph, Eppendorf<sup>TM</sup> and the pimonidazole binding assay. Glucose metabolism was evaluated by  $^{18}\text{F}$ -FDG autoradiography and compared with the pimonidazole binding distribution pattern. Repeated blood samplings significantly reduced mean haemoglobin levels ( $10.9 \pm 2.0 \text{ g/dl}$ ), inducing chronic anaemia in mice, while daily administration of rHuEPO led to increase of haemoglobin levels ( $15.8 \pm 2.0 \text{ g/dl}$ ). Oxygenation status evaluated by a microelectrode was worsened in anaemic mice (mean  $\text{pO}_2$

in tumour =  $6.9 \pm 0.8 \text{ mmHg}$ ) and improved in rHuEPO-treated animals (mean  $\text{pO}_2$  in tumour =  $11.4 \pm 1.2 \text{ mmHg}$ ). No correlation was observed between the oxygen-sensitive probe and pimonidazole labelling results: both techniques give different but complementary information about tumour hypoxia. Areas of high pimonidazole binding and areas of high  $^{18}\text{F}$ -FDG uptake superimposed well. Present results confirm that modification of haemoglobin levels leads to alteration of tumour oxygenation status. These variations were detectable using the oxygen-sensitive electrode but not the pimonidazole binding assay. The strong correlation between pimonidazole labelling and  $^{18}\text{F}$ -FDG uptake suggests a positive relationship between hypoxia and increased glucose metabolism in this tumour model.

## Introduction

Hypoxia is widely recognized as an intrinsic characteristic of a large variety of human solid tumours: head and neck (1) and uterine cervix carcinomas (2), breast cancers (3), soft tissue sarcomas (4), malignant brain tumours (5,6). Clinical studies based on microelectrode  $\text{pO}_2$  measurements have demonstrated that tumour hypoxia adversely affects prognosis (7-15). Poor tumour oxygenation promotes more aggressive tumour phenotypes, with a high probability of lymphatic spread and recurrence (10,11), and causes resistance to radiation therapy (7,9,15). Thus, detecting and overcoming tumour hypoxia are crucial aspects that could improve cancer treatment.

Several methods have been developed to assess hypoxia in xenografts and patient tumours. The gold standard is the use of a polarographic oxygen-sensitive probe, which provides direct measurement of tissue oxygenation status (16). Nevertheless, this invasive technique can only have limited clinical applications for superficial, thus accessible lesions. The 2-nitroimidazole compounds, such as pimonidazole and EF-5, are exogenous markers of hypoxia, and have been shown to

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be robust and effective markers of tissue hypoxia in both animal and human studies (17-21). After their biochemical reduction, these compounds bind selectively and irreversibly to macromolecules in viable hypoxic cells (19,22,23). A strong correlation, between the degree of hypoxia and the intensity of intracellular binding of the 2-nitroimidazole compounds (23,24) was demonstrated *in vitro* and *in vivo*. The 2-nitroimidazole intracellular binding can be detected by several techniques, such as immunohistochemistry and autoradiography which provide hypoxia evaluation at micro-regional level with a high spatial resolution, focusing on viable cells only, as dead cells do not metabolize these compounds.

$^{18}\text{F}$ -FDG, a radiolabelled glucose analogue, is commonly used in oncology imaging with positron emission tomography (PET) to detect primary lesions and their metastases. Its use is based on the increase of glucose metabolism in most solid tumours (25). As hypoxic cells often demonstrate enhanced anaerobic glycolysis, by increasing expression of glucose transporters and hexokinase activity (25), to maintain production of energy, it was hypothesized that  $^{18}\text{F}$ -FDG uptake could be an interesting and available method to detect indirectly tumour hypoxia.

To our knowledge, no study designed to compare hypoxia measurement methods has evaluated yet their sensitivity to detect *in vivo* weak variations of tumour hypoxia. This point is of particular importance when tumour radiosensitivity is investigated. Specifically, according to a review by Vaupel *et al.*, weak alterations of tumour oxygenation in tumours exhibiting low  $\text{pO}_2$  are sufficient to induce marked radiosensitivity variations (26).

Anaemia is one major parameter causing tumour hypoxia (26,27). Experimental and clinical data have provided evidence of a strong relationship between decreased haemoglobin levels and a poor oxygenation status in solid tumours (28-30). Conversely, the administration of recombinant human erythropoietin (rHuEPO) boosts erythrocyte synthesis and permits to overcome tumour hypoxia, as demonstrated in our previous work (31).

Therefore, to compare the sensitivity of hypoxia assessment methods, extreme conditions, i.e. chronic anaemia and erythropoiesis stimulation, were provoked to cause variations of tumour oxygenation. Experiments were performed in human glioma bearing-mice exhibiting different haemoglobin levels and hypoxia was assessed using both the polarographic oxygen-sensitive electrode which is the reference technique and pimonidazole binding revealed by immunohistochemistry. We also examined the relationship between tumour hypoxia and glucose metabolism, assessed by  $^{18}\text{F}$ -FDG autoradiography.

## Materials and methods

**Animals and tumour models.** Six- to eight-week-old, pathogen-free, athymic Swiss *nude* (*nu/nu*) mice were obtained from Charles River (St. Germain sur l'Arbresle, France). Animal procedures were performed according to institutional and national guidelines (EC directive 86/609/CEE). All experiments were carried out under general anaesthesia (i.p. injection of xylazine 8 mg/kg and ketamine 60 mg/kg).

The GBM Nan1 tumour used in this study, is a model of human malignant glioma previously described (31,32). Pieces of patient tumour were directly transplanted into *nude* mice. This tumour line was maintained *in vivo* by sequential passages in *nude* mice. The pathologic and immunohistochemical characteristics were unchanged with successive passages.

For the experiments, source tumours were excised, cleaned from necrotic tissue, cut into small fragments, and transplanted subcutaneously in either of the hind legs of each mouse. Twenty-four hours after tumour implantation, animals were randomized into three groups: the 'Control' group, where mice received no treatment, the 'Anaemia' group and the 'rHuEPO' group.

To induce anaemia, blood samples of 200  $\mu\text{l}$  were successively taken from the tail vein of mice by transecting the last few millimetres. The first blood sampling was performed 3 days after tumour implantation, the following every 8 days. Three to 4 blood samples were taken from each mouse until death.

To stimulate erythropoiesis in the 'rHuEPO' group, mice were injected with rHuEPO (Eprex<sup>®</sup>, Ortho-Biotech, Issy-les-Moulineaux, France). rHuEPO was diluted in sterile water to a final concentration of 30 IU/ml and was subcutaneously injected daily at a dose of 0.3 IU/g of body weight, 5 days per week. rHuEPO injections started 3 days after tumour implantation.

**Tumour growth.** Tumour size was determined 3 times per week by measuring the two largest perpendicular diameters with a calliper. Tumour volume ( $V$ ) was calculated as:  $V = x \text{ larger diameter} \times (\text{smaller diameter})^2$ .

When the target volume ( $300 \pm 100 \text{ mm}^3$ ) was achieved,  $\text{pO}_2$  measurements were performed. Then animals were sacrificed and tumours were excised and used for the following experiments.

**Measurement of haemoglobin concentration.** Blood samples were taken from the tail vein of mice by transecting its last few millimetres. Blood was drawn into a small capillary tube (Clinitubes, Radiometer, Neuilly Plaisance, France). Then, haemoglobin concentration was measured using a multiparameter automated haematology analyzer (Micros 60<sup>TM</sup>, HoribaABX, Montpellier, France).

In the 'Anaemia' group, haemoglobin level was evaluated 3 days after tumour implantation, when the first 200  $\mu\text{l}$  blood sample was withdrawn, then the day of animal sacrifice. In the 'Control' and 'rHuEPO' groups, haemoglobin concentration was determined the day of sacrifice.

**Polarographic measurements of tumour oxygenation.** When tumour volume reached  $300 \pm 100 \text{ mm}^3$ , oxygen partial pressure ( $\text{pO}_2$ ) was assessed into the hind leg muscle (normal tissue) and in the tumour.  $\text{PO}_2$  measurements were performed polarographically using the  $\text{pO}_2$ -Histogram (Eppendorf, Hamburg, Germany). Anaesthetized mice were immobilized by taping limb extremities on a polystyrene plate, taking care not to induce ischemia on animal tissue.

The instrument calibration and probe insertion into the tissue were performed as previously described (31). Probe

progression was set to 0.5 mm forward step and to 0.3 mm retraction step for each reading, and automatic probe advancement was started after the  $pO_2$  values had stabilized. The oxygen tension was evaluated at least from 4 different electrode tracks per tumour; a minimum of 12 measurements per track was recorded. In muscle, 2-4 tracks with 9-12 measurements per track were registered. Mean  $pO_2$  values and percentages of  $pO_2$  values <10 mmHg were calculated using the  $pO_2$ -Pool (version 1.2) software provided by Eppendorf.

**Pimonidazole and  $^{18}F$ -FDG injections.** Pimonidazole hydrochloride (Hypoxyprobe™-1), and the specific IgG<sub>1</sub> mouse monoclonal antibody (Mab1 antibody) directed against protein adducts of reductively activated pimonidazole were purchased from Chemicon International, UK. The use of pimonidazole is not expected to have effect on tissue  $pO_2$  measurements.

Four to six hours after  $pO_2$  measurements, mice were injected intraperitoneally with a dose of 60 mg/kg of pimonidazole hydrochloride, followed by a second injection 30 min later. Simultaneously to the first pimonidazole delivery,  $^{18}F$ -FDG (Schering, Vandoeuvre-lès-Nancy, France) was administered i.p. at a dose of 1.2 MBq/g of body weight. One hour after pimonidazole and  $^{18}F$ -FDG injections, mice were sacrificed, and tumours were immediately excised and frozen.

**$^{18}F$ -FDG autoradiography and pimonidazole immunohistochemistry.** Two histological slices were obtained from each frozen tumour using a cryostat Leica CM3050 S (Leica Microsystems SAS, Rueil-Malmaison, France): one 14- $\mu$ m thick slice was air dried at room temperature and used for both pimonidazole and  $^{18}F$ -FDG analysis, one adjacent 5- $\mu$ m thick slice was fixed in formalin and stained with haematoxylin and eosin for histopathologic observation, to confirm the presence of tumour, and to distinguish necrotic areas and non-tumoural tissue (e.g. stroma, inflammatory cells). The 14- $\mu$ m thickness was chosen in accordance with preliminary results showing that both the  $^{18}F$ -FDG autoradiographic analysis and the immunohistochemical pimonidazole binding analysis could be performed on the same tissue section without distorting histological observation (data not shown).

Immediately after histological slices were obtained, the 14- $\mu$ m thick slices were exposed for 90 min on the imaging plate of a  $\mu$ Imager™ (Biospace Mesures and Biospace Instruments SA, Paris, France), that allows recording of high-resolution images (<50  $\mu$ m) from  $\beta$  emitters.

After autoradiographic image acquisition, the slices were immunostained to reveal pimonidazole binding. The pimonidazole-induced protein adducts were detected using the primary Mab1 antibody (dilution 1:50). Revelation was based on immunoperoxidase techniques and to minimize non-specific immunostaining, the Zenon™ Mouse IgG labelling kit Z-25052 (Molecular Probes, Cergy Pontoise, France) was used according to the manufacturer's guidelines. Briefly, the biotin-labelled Fab fragment of the Zenon™ kit was pre-incubated with the primary Mab1 antibody to form a labelling complex and excess of Fab fragment was neutralized

by the addition of a non-specific IgG. The labelling complexes were applied on formalin-fixed tumour sections. Detection of tissue-bound biotinylated complexes was performed using the streptavidin-peroxidase system and bound peroxidase was identified using the Novared™ kit (Vector Laboratories Inc., Burlingame, CA, USA). Nuclei were counterstained with haematoxylin.

**Image analysis of pimonidazole labelling.** For qualitative analysis, immunostaining was considered when cells were identified as labelled above background by visual inspection, with no distinction between light or heavy immunostaining, assuming that all labelled cells irrespective of immunostaining intensity were at  $pO_2$  <10 mmHg (19,33), and were, therefore of interest with respect to increased radiation resistance. The pattern of pimonidazole binding was compared to histological parameters such as blood vessels and necrotic areas.

For quantitative analysis, we used a calibrated semi-quantitative scoring system developed by Raleigh *et al*, adapted by Nordsmark *et al*, and presenting an excellent interobserver reproducibility (34). As previously described (12,34), multiple fields per tumour section (mean 26; range 5-51, depending on section size and extent of necrotic areas) were analyzed with a light microscope at magnification x200. For each field, mean percent of the area immunostained was estimated as the percentage of the total area minus areas of acellularity, necrosis and stroma. Fields were assigned semiquantitative scores of 1+, 2+, 3+, and 4+, if the area fraction labelled fell in the ranges of 0-5%, >5-15%, >15-30% and >30%, respectively. Once scored, hypoxia was defined for each individual tumour as the percentage of fields at each score category.

**Autoradiographic image analysis - comparison with pimonidazole staining.**  $^{18}F$ -FDG uptake in tumours was evaluated by visual inspection. The regional distribution patterns of pimonidazole labelling and  $^{18}F$ -FDG uptake were directly compared using image co-registration.

Images of each immunostained tumour sections were captured at magnification x2.5 by means of an Axioskop 2 (Zeiss, Germany) linked through a high resolution camera (3.5  $\mu$ m/pixel) to a workstation running image software (Axiovision 4.5, Zeiss, Germany).

The stored images of both pimonidazole labelling and  $^{18}F$ -FDG uptake were rebinned to the same pixel size (50  $\mu$ m/pixel) and were co-registered, and merged in Adobe Photoshop (Adobe System Inc., San Jose, CA, USA). This allowed a direct and immediate comparison of the regional distribution of these two markers. Haematoxylin and eosin treated slides were used as references to define tumour tissue landmarks and to precise non-tumour tissue environment.

**Statistical analysis.** In the 'Anaemia' group, initial and final haemoglobin concentrations were compared using the non-parametric Wilcoxon rank test. The non-parametric Mann-Whitney U test was applied to compare mean  $pO_2$  and hypoxic fraction <10 mmHg, and also mean haemoglobin concentrations between the three experimental groups.



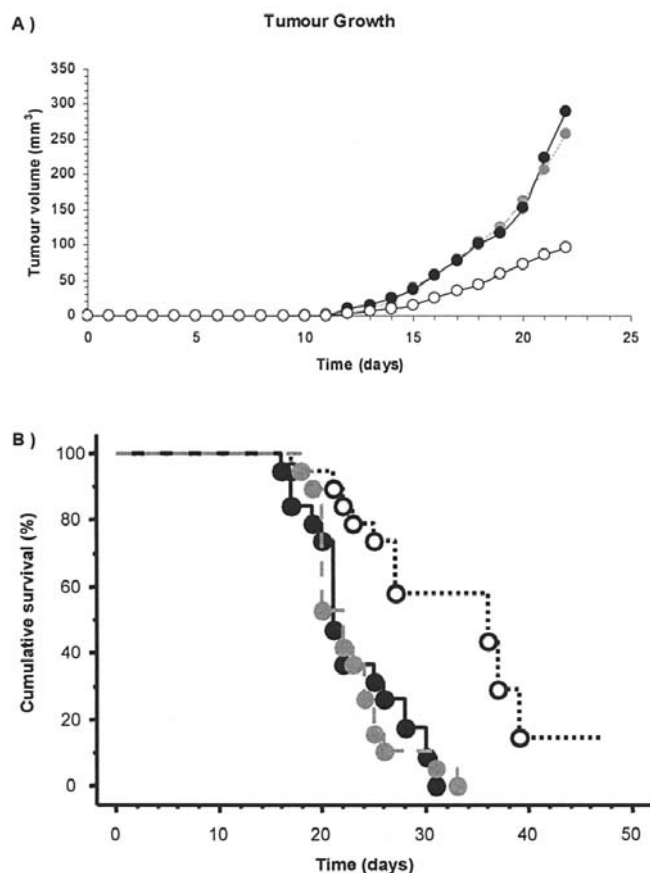


Figure 1. Evaluation of tumour growth. For anaemic mice ( $n=19$ ; grey circle), for control mice ( $n=19$ ; black circle) and for rHuEPO group ( $n=19$ ; empty circle), tumour size was measured 3 times per week, with a calliper until tumours reached a volume of  $300\pm 100\text{ mm}^3$ . (A) The graph represents mean tumour volume from the day of tumour implantation (day 0). (B) Kaplan-Meier survival curves.

Comparative tumour growth curves were prepared according to the Kaplan-Meier model, considering the percentage of tumours not having reached the target volume ( $300\pm 100\text{ mm}^3$ ). Statistical analysis was conducted through the log-rank test. For all tests used,  $p$ -values  $<0.05$  were considered significant.

## Results

**Determination of haemoglobin concentrations.** For the 'Anaemia' group, 3-4 successive blood samplings allowed to significantly reduce mean haemoglobin levels from  $14.8\pm 1.6\text{ g/dl}$  to  $10.9\pm 2.0\text{ g/dl}$  ( $p=0.012$ ), i.e. a mean decrease of 26% between initial and final haemoglobin concentrations. Considering each individual mouse, the extent of this decrease varied from 12 to 46%.

When tumours reached the target volume of  $300\pm 100\text{ mm}^3$ , corresponding to the day of sacrifice, blood haemoglobin levels were assessed for each experimental group. At sacrifice, haemoglobin levels were  $12.8\pm 1.5\text{ g/dl}$  in the untreated 'Control' group versus  $10.9\pm 2.0\text{ g/dl}$  in 'Anaemia' group ( $p=0.052$ ). Thus, repeated blood samplings resulted in a decrease of about 15% as compared with the 'Control' group.

Between tumour implantation and sacrifice, rHuEPO-treated mice were exposed to rHuEPO for 3-4 weeks. At the end of the experiment, a significantly higher haemoglobin level was recorded in the 'rHuEPO' group as compared with the 'Control' group ( $15.8\pm 2.0\text{ g/dl}$  vs  $12.8\pm 1.5\text{ g/dl}$ ;  $p=0.018$ ) or with the 'Anaemia' group ( $p=0.006$ ). Actually, daily rHuEPO injections led to a 23% increase of haemoglobin levels (vs 'Control' mice).

**Effects of chronic anaemia and rHuEPO on tumour growth.** Tumour volume was determined three times per week until the target volume of  $300\pm 100\text{ mm}^3$  was achieved. Tumour growth data are shown in Fig. 1. Whereas chronic anaemia did not alter tumour growth, this latter was significantly delayed in rHuEPO-treated mice as compared with the 'Anaemia' ( $p=0.002$ ) and 'Control' ( $p=0.03$ ) groups.

**Effects of chronic anaemia and rHuEPO on muscle oxygenation and on tumour hypoxia.** In the three experimental groups, the oxygen partial pressure was determined in normal tissues (hind leg muscle) and in tumours. The results presented in Table I are expressed as mean  $p\text{O}_2$  and hypoxic fraction  $<10\text{ mmHg}$  (i.e. percentage of  $p\text{O}_2$  values  $<10\text{ mmHg}$ ), and show that the oxygenation status in muscle was worsened in anaemic nude mice and improved in rHuEPO-treated animals, as compared with the 'Control' group. Furthermore, mean

Table I. Effects of chronic anaemia and rHuEPO treatment on oxygenation status in muscles and tumours.

	Muscle			Tumour		
	n	Mean $p\text{O}_2$ (mmHg)	HF $<10\text{ mmHg}$ (%)	n	Mean $p\text{O}_2$ (mmHg)	HF $<10\text{ mmHg}$ (%)
Anaemia	6	$8.9\pm 2.5$	$68.7\pm 13.6$	10	$6.9\pm 0.8$	$82.7\pm 3.5$
Control	12	$14.0\pm 1.8$	$41.2\pm 11.0$	10	$8.6\pm 0.8$	$75.3\pm 4.1$
rHuEPO	8	$17.1\pm 2.9^a$	$31.7\pm 12.0^a$	9	$11.4\pm 1.2^{a,b}$	$53.5\pm 9.1^{a,b}$

Results are expressed as 'mean  $\pm$  standard error'. n, number of mice per group; HF, hypoxic fraction. <sup>a</sup>Difference statistically significant between 'rHuEPO' group and 'Anaemia' group (Mann-Whitney U test;  $p<0.05$ ). <sup>b</sup>Difference statistically significant between 'rHuEPO' group and 'Control' group (Mann-Whitney U test;  $p<0.05$ ).

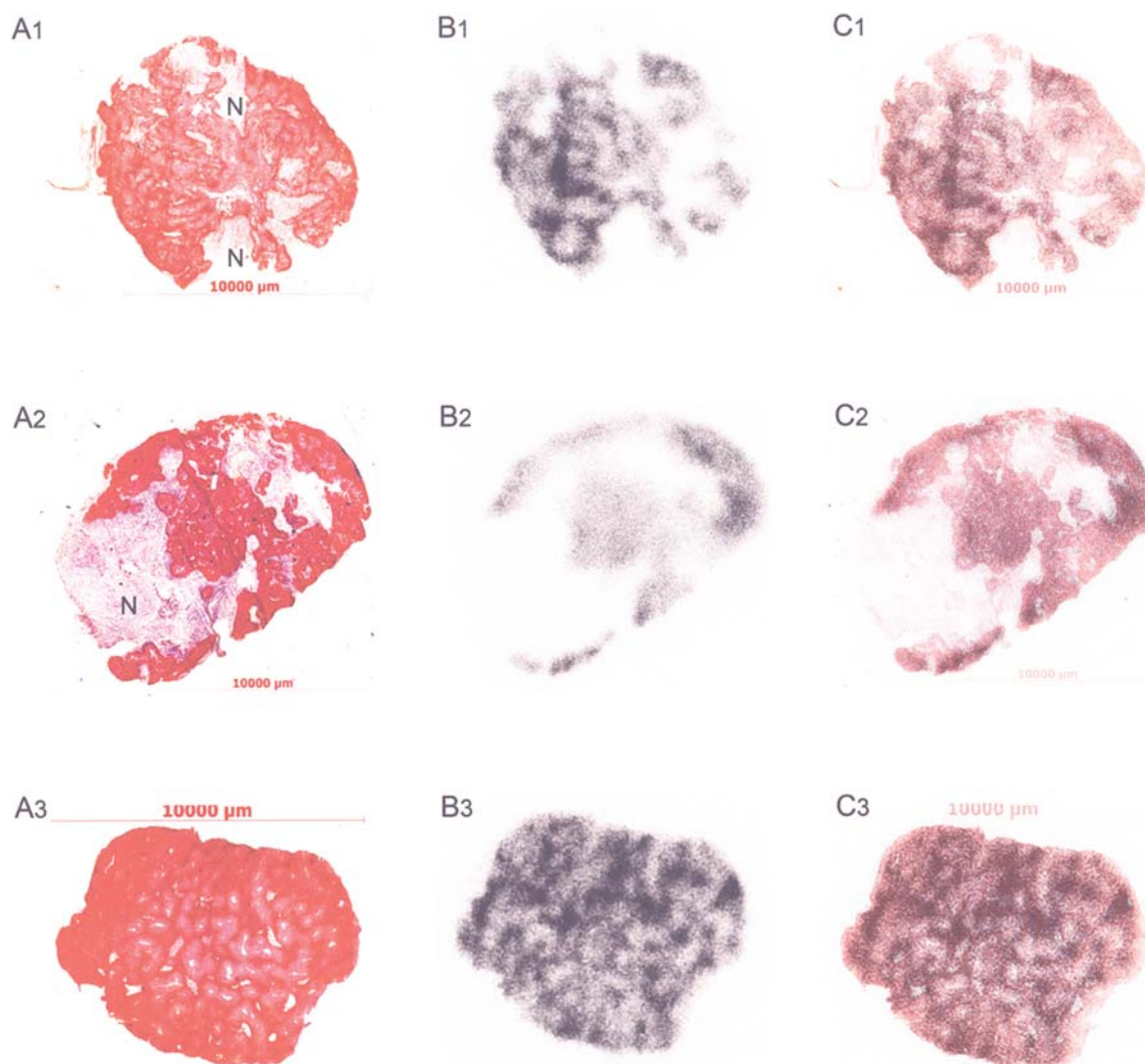


Figure 2. Pimonidazole immunostaining and  $^{18}\text{F}$ -FDG radiolabelling. The same tumour section was immunostained to reveal pimonidazole binding (A) and imaged for  $^{18}\text{F}$ -FDG uptake using the  $\mu$ Imager (B). The stored images of both pimonidazole labelling and  $^{18}\text{F}$ -FDG uptake were co-registered to allow a direct and immediate comparison of the regional distribution of these two markers (C). A representative xenografted tumour for each group is presented: anemia group (A1, B1, C1), control group (A2, B2, C2), and rHuEPO group (A3, B3, C3). Images show a great heterogeneity of parameters, with good superimposition of areas of high pimonidazole binding and high  $^{18}\text{F}$ -FDG uptake. Regions of necrosis (N) were not immunostained and did not show  $^{18}\text{F}$ -FDG uptake.

$\text{pO}_2$  and hypoxic fraction  $<10$  mmHg were statistically different between the 'Anaemia group' and the 'rHuEPO' group ( $p=0.045$  and  $0.033$ , respectively).

In tumours, similar trends were observed. Actually, repeated blood samplings led to a 20% decrease of mean  $\text{pO}_2$  and a 10% increase of hypoxic fraction  $<10$  mmHg, as compared with the 'Control' group. rHuEPO exposure induced a statistically significant increase of mean  $\text{pO}_2$  of about 33% ( $p=0.018$  vs 'Control' group) and a decrease of the hypoxic fraction of about 29% ( $p=0.050$  vs 'Control' group).

**Pimonidazole labelling.** In each experimental group, each tumour section was found to contain hypoxia, i.e. to show presence of areas of pimonidazole binding (Fig. 2A). Patterns and absolute levels of pimonidazole binding substantially varied in the same tumour and between the tumours of the same group. There was no staining in necrotic regions, and

only minimal non specific staining in non-tumour tissues. Pimonidazole labelling was present in cells surrounding necrotic areas, and occurred in most cases far from blood vessels, which was consistent with chronic hypoxia (diffusion limited oxygenation). Less frequently, it also occurred close to blood vessels, which was consistent with acute hypoxia (perfusion limited oxygenation).

Because of the highly heterogeneous distribution pattern of pimonidazole binding, no difference between the three experimental groups was observed (Fig. 3A). In each group, ~50% of the observed fields were scored 4+, indicating that GBM Nan1 glioma xenografts are very hypoxic.

Data obtained from oxygen electrode measurements and from pimonidazole binding were compared (Fig. 3B). For this, the relationship between the percentage of microscopic fields at score 4+ and the hypoxic fraction  $<10$  mmHg was studied. Widespread values were observed, indicating no correlation between these two parameters of tumour hypoxia.

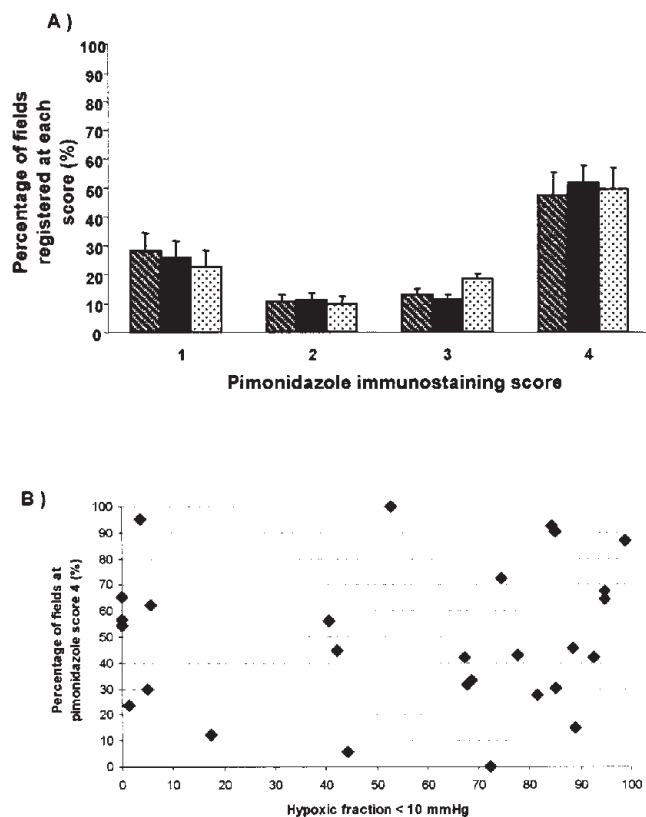


Figure 3. Pimonidazole immunostaining. A calibrated semi-quantitative score to quantify pimonidazole binding was used as previously described (12,34); scores, based on a 1+, 2+, 3+, 4+ scale, represent the ratio (%) between pimonidazole-immunostained area and total area of the observed field and correspond to 0-5%, 5-15%, 15-30% and >30%, respectively. (A) Histograms representing the proportion (%) of fields registered at each score for anaemia group (n=12; hatched square), for control group (n=18; black square), for rHuEPO group (n=16; black dotted square). (B) The percentage of microscopic fields at pimonidazole score 4 as function of the hypoxic fraction <10 mmHg (n=28 tumours).

**Comparison between pimonidazole labelling and  $^{18}\text{F}$ -FDG autoradiography.** The relationship between tumour hypoxia and glucose metabolism was assessed by comparing the distribution patterns of pimonidazole binding and  $^{18}\text{F}$ -FDG uptake.

Similarly to pimonidazole immunostaining,  $^{18}\text{F}$ -FDG uptake was heterogeneously distributed within and between tumours in each experimental group (Fig. 2B). No or low  $^{18}\text{F}$ -FDG uptake was present in necrotic areas. Increased  $^{18}\text{F}$ -FDG uptake was usually present in the border of necrotic regions, but foci of high  $^{18}\text{F}$ -FDG uptake also occurred in areas far from necrotic regions. No obvious difference (distribution pattern or uptake intensity) was observed between the three experimental groups (Fig. 2B1, 2B2 and 2B3).

In Fig. 2C, image co-registration analysis confirmed that areas of high pimonidazole binding and areas of high  $^{18}\text{F}$ -FDG uptake superimposed well, suggesting that in the glioma xenografts studied, there was a good positive correlation between hypoxia and increased glucose metabolism. This concordance was observed in almost every tumour section.

## Discussion

Among the diverse methods of quantifying tumour hypoxia, direct  $\text{pO}_2$  measurements with a polarographic oxygen-sensitive electrode is considered as the gold standard (16). The use of hypoxic cell markers such as pimonidazole is also a well established method for assessing oxygenation both in xenografts and human tumours (16-21).

The first purpose of this work was to compare in human malignant glioma-bearing mice the sensitivity of these two techniques, especially their ability to detect expected weak variations of tumour hypoxia. To favour such modifications and because of the known relationship between haemoglobin levels and tumour oxygenation (35), it was chosen to induce variations of haemoglobin concentration. In cancer patients, anaemia is associated with poor tumour oxygenation (28-30). In animal models the administration of recombinant human erythropoietin (rHuEPO) increases haemoglobin levels, and thus permits to overcome tumour hypoxia, as demonstrated in our previous work (31). In the present study, anaemia was induced by repeated blood samplings: this method was used to avoid any potential general effect, particularly on tumour growth as it may be expected when chemotherapy or total body irradiation is used. In the 'Anaemia' group, we observed a mean haemoglobin level decrease of 15% as compared with the 'Control' group. Conversely, to increase haemoglobin levels, we injected rHuEPO to the animals 5 days per week and we observed an increase of haemoglobin levels of about 20-25% after 3-4 weeks of this administration schedule. These alterations of the oxygen-carrying capacity of the blood were associated with changes in tissue oxygenation status, when measured with the oxygen-sensitive probe: oxygen partial pressure measured in muscle (normal tissue) and in glioma xenografts was decreased in anaemic mice and increased in rHuEPO-treated animals. These results are consistent with our previous published data demonstrating an rHuEPO-induced reduction of tumour hypoxia in two models of human malignant glioma (GBM Nan1 and U87) (31). It is noteworthy that in this glioma model, tumour growth was slowed down in rHuEPO-treated mice, whereas several reports raised the possibility of tumour growth stimulation by rHuEPO (36,37). Our observation confirms recent data indicating that rHuEPO exerts no stimulatory effect on tumour growth evaluated on four tumour cell lines *in vivo* (38).

The pimonidazole binding assay was proposed to replace the oxygen-sensitive electrode method, because the latter can only be applied to tumours accessible from the body surface. Furthermore, pimonidazole was already demonstrated to be able to detect hypoxia differences when chemotherapy-induced anaemia was corrected by darbepoietin alpha in murine tumours (39). In our experiments, both the oxygen-sensitive probe and pimonidazole binding assay permitted to identify tumour hypoxia, but only the former allowed to detect tumour oxygenation differences between 'Anaemia' and 'rHuEPO' groups. One explanation for this discrepancy could be that our xenograft model was much more hypoxic than that used by Shannon *et al.* (39). The oxygen-sensitive probe and pimonidazole binding have been previously compared with contradictory results: Nordmark *et al.*



reported no significant correlation between the two assays in human cervix carcinomas (12,40), while Raleigh *et al* showed a good correlation between hypoxia evaluated by pimonidazole-binding assay and  $pO_2$  measurements in a C3H mouse mammary carcinoma (33). In our glioma model, we found no correlation between the fraction of fields immunostained at the highest score and the  $pO_2$  measurements. It is noteworthy that our glioma model and the human cervix carcinomas evaluated by Nordsmark *et al* (12,40) were markedly hypoxic and the semiquantitative scoring system developed by Raleigh *et al* (34) may not be discriminant enough to observe weak variations of oxygenation status in very hypoxic tumours, as was the case between our three experimental groups. Actually, the hypoxic fraction <10 mmHg represented >70% in our 'Control' group, and 72% in the human cervix carcinomas investigated by Nordsmark *et al* (12,40) versus only 57% in the experiments of Raleigh *et al* (33). The semi-quantitative scoring system used also showed that GBM Nan1 glioma xenografts were very hypoxic, but as it did not differentiate between high and low intensities of pimonidazole binding, and did not distinguish microscopic fields when the labelled area fraction was >30%, it failed to show differences between the three experimental groups.

According to the present results, the pimonidazole binding assay and oxygen-sensitive electrode measurements appear more complementary than competitive methods, and both methods give relevant information about intratumoural hypoxia. The oxygen-sensitive probe provides a global, instantaneous value obtained from a large number of measurements. This quantitative value is important to characterize a given tumour, and is useful to correlate hypoxia with general parameters such as response to radiation therapy and survival (7-15). The parameter measured is predominantly the extracellular oxygen tension, and because the electrode localization within tumours can not be fully controlled during  $pO_2$  measurements, it is not possible to distinguish between measurements performed in necrotic or hemorrhagic areas, or in non-tumour areas, that leads to overestimation (in the case of necrotic areas) or underestimation (in the case of haemorrhagic areas) of hypoxic values.

With pimonidazole binding assay, hypoxia is observed on a cell to cell basis, and only viable hypoxic tumour cells are considered, since necrotic cells can not metabolize the drug. The immunohistochemical detection of pimonidazole binding gives the microregional distribution of hypoxia that can not be characterized by a single value. Data about the intratumoural distribution of hypoxia are of importance in experimental preclinical or clinical trials to understand the various aspects of tumour biology, as immunohistochemical pimonidazole binding can be easily compared with other physiological or pathological parameters, such as cellular proliferation, cell death, and vasculature.

Nordsmark *et al* (12,40) noted the distinct time resolution between electrode measurements and hypoxic markers: while oxygen electrodes provide an instantaneous measure of tumour hypoxia, pimonidazole binding provides a measure of hypoxia integrated over a longer period (the period of drug exposure), measuring thus effects of both acute and chronic hypoxia and being more sensitive than  $pO_2$  measurements to measure chronic hypoxia.

Zimny *et al* (41) studied 24 patients with head and neck malignancies, and compared the results of tumour hypoxia assessed by  $pO_2$  measurements and by  $^{18}F$ -FMISO, a radio-labelled nitroimidazole imaged by positron emission tomography (PET). For comparison with  $pO_2$  measurements, they chose the tumour to blood ratio of  $^{18}F$ -FMISO uptake and found a strong correlation ( $r=0.80$ ,  $p<0.001$ ) between the oxygenation results obtained from the two methods. But, they observed a high discrepancy in two patients with mostly necrotic lymph node metastases and only a small amount of vital tumour tissue, thus indicating limitations for agreement of both methods because of the methodological differences noted above.

In the second part of this study, we examined the spatial relationship between tumour hypoxia assessed by immunohistochemical pimonidazole binding and intratumoural glucose metabolism imaged by  $^{18}F$ -FDG autoradiography. Substantial experimental data show that hypoxia leads to increased glucose consumption, thus increased  $^{18}F$ -FDG uptake, via increased expression or modified forms of glucose transporters and increased hexokinase activity (25,42-44). In this GBM Nan1 glioma xenograft, we observed a strong concordance between areas of high pimonidazole staining and areas of high  $^{18}F$ -FDG uptake, suggesting that an increased glucose metabolism occurred in the more hypoxic cells. Although *in vitro* in human cancer cell lines the positive relationship between increased cellular  $^{18}F$ -FDG uptake and hypoxic conditions is well established (42-44), *in vivo* discordant results have been reported. Dearling *et al* (45) reported increased  $^{18}F$ -FDG uptake in the hypoxic regions of human tumour xenografts, when compared with that in normoxic regions and Pugachev *et al* (46) reported a positive correlation between  $^{18}F$ -FDG uptake and hypoxia assessed by pimonidazole in *nude* mice bearing prostate tumours. Conversely, Rajendran *et al* (47) and Zimny *et al* (41) showed that hypoxia and glucose uptake are not systematically positively correlated, depending on the histological tumour type, and consecutively on the intrinsic intratumoural microenvironmental conditions affecting diversely glucose metabolism.

In conclusion, the present study shows that modifications of oxygen-carrying capacity of the blood influence the oxygen status in non-tumoural (muscle) and tumoural tissue, when measured globally with oxygen-sensitive probes: an induced chronic anaemia increases tissue hypoxia, whereas daily administration of rHuEPO, via the increase of haemoglobin concentration, reduces intrinsic hypoxia, without accelerating tumour growth in our animal model. Our experiments failed to find a correlation between oxygen-sensitive probe measurements and pimonidazole binding, but highlighted the complementarity of the two techniques to understand and analyze more precisely tissue hypoxia. Although pimonidazole staining and  $^{18}F$ -FDG uptake were not discriminant enough to distinguish low variations of oxygen tension, distribution pattern of both methods superimposed well in our glioma model. Because of many discrepancies between results in the literature, further investigations are needed to determine whether  $^{18}F$ -FDG is a suitable indirect hypoxia marker.

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