Photodynamic therapy for malignant pleural mesothelioma
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OXYGEN DEPLETION DURING AND AFTER mTHPC MEDIATED PHOTODYNAMIC THERAPY IN RIF1 AND H-MESO1 TUMORS

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ABSTRACT

During photodynamic therapy (PDT) low oxygenation levels, induced both by oxygen consumption and by vascular occlusion, can lead to an inefficient photochemical reaction which may compromise PDT efficacy. In the present studies tumor oxygenation was measured before, during and after meta-Tetrahydroxyphenylchlorin (mTHPC) mediated PDT for RIF1 tumors and a human mesothelioma xenograft model (H-MESO 1) in nude mice. Tumor tissue oxygenation was measured in real time with Eppendorf polarographic histography. Decreased mean median values, as well as an increase in the number of values below 2.5 mm Hg, indicated that hypoxia was induced during and after PDT in RIF1 tumors. Tumor pO2 values did not change significantly in H-MES01 tumors. Staining with antibodies against the hypoxic marker EF3, however, showed significant increases of the hypoxic fraction directly after and 60 minutes after PDT when compared with untreated controls in both tumor types. Extensive necrosis may have prevented the detection of PDT induced hypoxia using the Eppendorf polarographic needle.

Conclusion: We have demonstrated oxygen depletion during and after mTHPC mediated PDT in RIF1 and H-MESO1 tumors with the hypoxia marker EF3 and by polarographic needle measurements for RIF1 tumors but not for H-MESO1 tumors. Considerable amounts of necrosis may influence the usefulness of the polarographic technique for measuring changes in oxygenation status in some tumors.

INTRODUCTION

Photodynamic therapy (PDT) is being used successfully for the treatment of small superficial tumors, mainly in the oral cavity (1,2), the esophagus, the bronchial tract (3,4) and on the skin (5-7). Another application is its use as an adjuvant treatment in combination with surgery for more widespread disease, such as malignant mesothelioma (8-12).

For effective and safe PDT, parameters such as type and concentration of the photosensitizer, drug-light interval, light intensity (fluence rate) and total light dose (total fluence), should all be chosen with respect to indication and PDT related conditions such as backscattering (2,10). Another factor important for PDT, but less easy to standardize, is the oxygenation status of the tumor. Oxygen is a prerequisite for the photochemical reactions and insufficient availability of oxygen reduces the efficiency of PDT. During the photochemical reactions oxygen is consumed and free radicals and the highly toxic singlet oxygen, which cause cell death, are produced (13). In addition to direct cell kill, PDT induces extensive vascular damage resulting in vasoconstriction and vessel occlusion, which lead to secondary tumor hypoxia and destruction. PDT induced tumor hypoxia can result both from oxygen consumption during the photochemical reaction and as a consequence of diminished supply of oxygen due to blood flow impairment. Both types of hypoxia have been clearly demonstrated in a rabbit skin tumor model which was illuminated with intermittent illumination after sensitization with a hematoporphyrin derivative (14). In this model, the oxygen tension of the tumors decreased during illumination (oxygen consumption) and recovered to baseline during dark periods. After 3 such cycles the oxygenation levels no longer recovered during dark periods as permanent vascular constriction developed due to PDT damage.

The relationship between oxygen consumption and the efficacy of PDT is influenced by the fluence rate and sensitizer dose. Oxygen depletion during PDT is more
likely to occur at high fluence rates with high sensitiser doses, where the photochemical reaction proceeds most rapidly (15-18). Vascular occlusion is dependent on the total fluence delivered and is usually maximal a few hours after the end of illumination, although partial or total vessel occlusion can already occur during prolonged PDT (19,20).

Oxygenation depletion during and after PDT using Photofrin as photosensitizer has previously been demonstrated in murine fibrosarcomas (RIF1) tumors (18). In this study we examined changes in oxygenation levels before, during and after PDT mediated by meta-tetrahydroxyphenylchlorin (mTHPC), which is one of the most potent photosensitizers in clinical use. PDT induced hypoxia was estimated from tumor oxygenation levels and the percentage of hypoxic cells in RIF1 and human mesothelioma xenografts (H-MESO1). The RIF1 tumor is well vascularized and almost free of necrosis, for tumors < 10 mm in diameter, in contrast to the H-MESO1 which has evident areas of necrosis even at small tumor sizes. Oxygenation levels during and after PDT were assessed with an Eppendorf polarographic histograph, which is the best validated technique to measure tissue oxygenation levels in real time. Extent and distribution of tumor hypoxia were also measured using immunohistochemical staining of tumor sections with the hypoxia marker EF3 (21).

MATERIALS AND METHODS

Animal models

All experiments were carried out in accordance with protocols approved by the local experimental animal welfare committee and they were conformed to national and European regulations for animal experimentation. Radiation Induced Fibrosarcoma cells (RIF1) of C3H/Km mice were maintained and passed according to recommended in vivo and in vitro protocols (22). Cells were inoculated subcutaneously (s.c.) \(1 \times 10^5\) cells per mouse) on the lower dorsum of female C3H/Km mice, which were briefly anesthetized with enflurane. Human malignant mesothelioma cells (H-MESO1, from R.F. Camalier, National Cancer Institute, Maryland, U.S.A.) were taken from frozen stock and grown in vitro, in RPMI medium supplemented with 10% FCS, prior to inoculation in nude mice. Cells were kept in culture for a maximum of 12 passages before returning to the frozen stock. To propagate tumors in vivo, \(5.10^6\) cells were injected s.c. in the flanks of female Balb/C nude donor mice. The tumors were allowed to grow to 8 to 10 mm diameter before excision of the donor tumor and transplantation of small fragments (diameter about 1 mm) to recipient experimental mice. Tumor fragments were transplanted s.c. on the lower dorsum (using a trocar) under ether anesthesia. Mice were used for experiments at an age of 11 – 16 weeks, weighing 21 to 30 grs. Tumors were treated, and hypoxia measurements made, when the mean tumor diameter had reached \(7 \pm 2\) mm.

Photodynamic therapy

mTHPC was injected i.v. at a dose of 0.3 mg/kg body weight, 24 hours before illumination. Superficial illumination of the tumors was performed with red light (652 nm) using a diode laser (Applied Optronics, South Plainfield, USA) and a microlens to deliver the light over a 15 mm diameter area encompassing the tumor. Power output was measured with an optical power meter (UDT 371 R) connected to an integrating sphere
(model 2500, Graseby Optronics, Orlando, USA) and tumors were illuminated at a fluence rate of 100 mW.cm\(^{-2}\), to a total light dose of 100 J.cm\(^{-2}\), total illumination time 16 minutes and 40 seconds.

**Tumor pO\(_2\) measurements**

Extensive details of the principles of pO\(_2\) measurement using the Eppendorf microelectrode were described by Vaupel et al (23). A brief description of these principles is given here. Oxygen partial pressure is measured by a 17 um gold polarographic microelectrode contained in the cavity of a 300-mm bevelled steel needle probe, covered by a Teflon membrane. The electrode is polarized to a Ag/AgCl anode with 700 mV, which is placed subcutaneously. Oxygen measurement is effected by electrochemical reduction of oxygen at the cathode. The resulting current is proportional to the oxygen partial pressure at the electrode tip.

Tumor bearing mice were anesthetized with hypnorm (0.8 mg.kg\(^{-1}\) i.p.) and midazolam (1.5 mg.kg\(^{-1}\) i.p.) and restrained on a flat surface before perforating the skin with a needle and inserting the electrode into the tumor. A total of 32 measurements were taken from four different tracks at one side of the tumor. The measurement path was 6 mm in length, with a step length of 0.7 mm, an overstroke of 0.3 mm and a response time of 1.4 second. Each period of measurements was alternated with a calibration period of 5 minutes. Oxygen measurements in each tumor were performed before, at 2 and 11 minutes after starting illumination, and at 5 and 60 minutes after terminating illumination. A separate control group was used to investigate the effect of multiple needle insertions, with measurements performed with 5 minute intervals.

All results of the pO\(_2\) measurements were expressed as median pO\(_2\) of each measurement period. The mean of these median values was used for further analysis. Negative values were included in the analysis; there were never more than 3.4% negative values and the lowest value was -7.6 mm Hg. The percentage of pO\(_2\) values less than 2.5 mm Hg was also determined, because oxygenation below this threshold is considered insufficient for effective PDT. (24).

**Immunohistochemistry for hypoxic tumor areas**

The hypoxic marker EF3 [2-(2-nitroimidazol-1[H]-yl)-N-(3,3,3-trifluoropropyl) acetamide], kindly supplied by C. Koch, (University of Pennsylvania, Philadelphia, U.S.A.) was dissolved in phosphate buffered saline (PBS), 5.33 mg.ml\(^{-1}\), and injected i.v. in a volume of 0.25 ml per mouse (~53 mg.kg\(^{-1}\)). Animals were injected 5 minutes before illumination and killed by cervical dislocation immediately after the end of illumination or 1 hour after illumination (i.e. 22 and 82 minutes after EF3 injection). These time spans were chosen for direct comparison with results obtained by Eppendorf Histography. Excized tumors were frozen immediately on dry ice and stored at – 80 OC until sectioning. Frozen sections were air dried and fixed in acetone. Non-specific sites were blocked with 10% normal donkey serum (Jackson immunoresearch Laboratories, West Grave, U.S.A.) in PBS. The blocking serum was applied together with a Cy3 labelled mouse anti EF3 antibody (again kindly supplied by C. Koch), in a concentration of 20 mgr.ml\(^{-1}\), and sections were incubated overnight at 40 C. Sections were washed for 30 minutes in PBS and covered with Fluorostab (ITK, Uithoorn, The Netherlands). After immunohistochemical staining for hypoxia, sections were scanned using an image analysis system. A filter
combination of 510 – 560 nm excitation and 590 nm emission was used. Fluorescence signals were recorded by a high resolution intensified solid state video camera (MXRi, HCS, The Netherlands). The video signal was digitized to images with 256 gray levels. The digital imaging application, TLC – Image (TNO, Delft, The Netherlands), was used for recording and further processing the images and controlling a motorized scanning stage (EK 32, Märzhäuser, Wetzlar, Germany) attached to a fluorescence microscope (Axioshop, Zeiss, 100 Watt short arc mercury lamp). Preceding each scan, the corresponding gray value image was loaded in computer RAM from the hard disk and the stored stage coordinates were used to move the stage to the starting point automatically. Next, the section was automatically rescanned, and hypoxic regions were detected using the previously determined threshold. All sections of the Cy3 labeled tumors were processed in one series. Major artefacts like air bubbles were excluded in both analyses. The relative hypoxic fraction was expressed as the total hypoxic area divided by the total tumor area. Two analyses were performed, one with and one without exclusion of areas of obvious necrosis in the adjacent sections stained with H&E. H&E and EF3 images were matched using the Adobe Photoshop 4.0.1. software program.

Statistical considerations

Tumor oxygenation data were analysed for differences per tumor as experimental conditions varied. Median tumor pO$_2$ and percentages of values < 2.5 mmHg were first logarithmically transformed to obtain a normal distribution of residuals. The covariance structure to be used in the repeated measurements ANOVA (RM-ANOVA) was then selected, based on the Bayesian Information Criterion, resulting in the choice of the CS model for the RIF1 tumors and the AR(1) model for the H-MES01 tumors. RM-ANOVA was used to calculate group means and standard errors; p-values were calculated from approximate type III F-tests. Slopes with their standard errors were calculated from the RM-ANOVA group means using ordinary linear regression with equi-distant time points.

Immunohistochemical data were analyzed for differences between pre- and post treatment measurements. Means of measured hypoxic fractions were log transformed before analysis to obtain a normal distribution and then analyzed with three-way analysis of variance (ANOVA) including all possible interactions.

RESULTS

Eppendorf measurements

Oxygen partial pressure in RIF1 and H-MES01 tumors was measured before, during and after PDT. Anesthesia with hypnorm and dormicum did not induce changes in mean median pO$_2$ or percentages values below 2.5 mmHg in RIF1 tumors (data not shown). The influence of repeated insertions of the Eppendorf probe and light only was measured in separate groups of RIF1 control tumors. Table 1 shows mean median pO$_2$ values of measurements made during repeated insertions of the probe in control tumors (tracks only and light only) and in PDT treated tumors. The mean median pO$_2$ values for untreated RIF1 tumors (tracks only) tended to increase with the number of measurements
### Table 1. Oxygenation of RIF1 tumours in relation to PDT

Mean median pO$_2$ in mm Hg ± STD

<table>
<thead>
<tr>
<th>Time point</th>
<th>Control tracks only</th>
<th>Control light only</th>
<th>PDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_1$</td>
<td>6.2 ± 7.1</td>
<td>5.4 ± 4.7</td>
<td>9.3 ± 6.0</td>
</tr>
<tr>
<td>$T_2$, illum.</td>
<td>7.8 ± 7.0</td>
<td>7.3 ± 4.6</td>
<td>8.5 ± 7.4</td>
</tr>
<tr>
<td>$T_3$, illum.</td>
<td>10.2 ± 6.3</td>
<td>12.5 ± 11.5</td>
<td>7.5 ± 6.5</td>
</tr>
<tr>
<td>$T_4$</td>
<td>10.8 ± 7.7</td>
<td>8.3 ± 3.3</td>
<td>8.7 ± 8.6</td>
</tr>
<tr>
<td>$T_5$</td>
<td>11.8 ± 11.1</td>
<td>5.4 ± 1.9</td>
<td>5.0 ± 4.2</td>
</tr>
<tr>
<td>% negative values</td>
<td>0.9</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>Most negative value</td>
<td>-3.4</td>
<td>-2.3</td>
<td>-0.9</td>
</tr>
</tbody>
</table>

Mice per group

|                         | 12 | 11 | 24 |

#### Repeated Measurements – ANOVA

| Slope ± SE | 0.156 ± 0.087 | 0.060 ± 0.047 | -0.146 ± 0.031 |
| p value slope | 0.076 | 0.21 | 0.0002 |

**Differences in slope between groups (p value)**

| PDT vs Tracks only | 0.0045 |
| PDT vs Light only  | 0.0026 |

$T_1$ = 5 minutes before start of illumination. $T_2$ and $T_3$ = 2 and 11 minutes after start of illumination, $T_4$ and $T_5$ = 5 and 60 minutes after end of illumination. In tracks only group: $T_{1-5}$ 5 minutes between measurements.
although this did not quite reach significance (p = 0.076). There was no evidence for a change of pO₂ with time in the tumors which received light only, and there was no significant difference between the tracks only and the light only groups (p = 0.89). For tumors treated with PDT, there was a significant decrease in mean median pO₂ with time (p = 0.0002). At T2 or T3, 19 out of the 24 tumors showed a decrease in pO₂ compared with T1, however, only 11 tumors had a lower median pO₂ at both T2 and T3. The overall change in pO₂ with time was predominantly due to a decrease 1 hour after PDT (T5). A comparison of the T1 values in the PDT group versus T1 values of the tracks only and light only groups revealed that the baseline pO₂ for the PDT group (i.e. 24 hours after injection of mTHPC but before illumination) was higher than in non sensitized tumors, but this difference did not reach significance.

Data for percentages of pO₂ values < 2.5 mmHg are shown in Figure 1, for the same groups of mice as were given in Table 1. Statistical analyses of these data lead to the same conclusions as for data derived from mean median pO₂ values, with respect to changes in tumor oxygenation. The tracks only group showed a non significant decrease in the percentage < 2.5 mmHg with time (p = 0.14), the light only group showed a temporary decrease during illumination, but no significant overall change (p = 0.65). PDT induced a significant increase of percentages values < 2.5 mmHg with time, again this was most striking 1 hour after illumination (p<0.0001).

Eppendorf mean median pO₂ values for the MES01 xenografts are shown in Table 2. There was no evidence for a change in mean median pO₂ with time in the tracks only control group (p = 0.57) or in the PDT group (p = 0.42). Although 8 out of the 12 tumors had a lower median pO₂ at T2 or T3, only 3 were lower at both T2 and T3 when compared with T1. The baseline values (T1) for the PDT group (i.e. 24 hours after mTHPC injection, but before illumination) were slightly lower than for the tracks only group, but this was not significant (p = 0.29). Percentages values below 2.5 mmHg (data not shown) also did not change significantly with time in the tracks only control group or in the PDT treated group (p = 0.41 and 0.56 respectively).

**Relative hypoxic fraction**

Hematoxylin Eosin staining of control and PDT treated RIF1 and H-MESO1 tumors demonstrated considerable differences in extent of necrosis. Before (Fig. 2A) and directly after PDT of RIF1 tumors there was hardly any evidence of necrosis. One hour after PDT, of RIF1 tumors 25 % of the total surface analyzed in 3 RIF1 tumors was visibly necrotic (not shown). In H-MESO1 tumors, large areas of necrosis (40 % or more) were clearly visible even before PDT (Fig. 2B). The extent of necrosis did not change significantly during the first hour after PDT in these tumors (not shown).

In untreated RIF1 tumors only a very small percentage of the non necrotic tumor area was labeled (indicative of hypoxia) at 22 and 82 minutes after EF3 injection (0.5% and 1% respectively, Figure 3, open bars). Although the percentages of EF3 binding are low, the considerable increase in hypoxic labelling with time from injection of EF3 indicates continuing bioreduction of the marker within this time frame. Comparisons of hypoxic fractions in controls and PDT treated tumors must therefore be done at equivalent times from injection of EF3. The hypoxic fraction of treated tumors increased significantly both immediately after illumination (22 minutes after EF3 injection) and 1 hour after illumination (82 minutes after injection, Figure 3 closed bars). Analysis of the hypoxic fraction without exclusion of necrotic areas revealed the same significant differences between control and
Figure 1

Percentages pO₂ values < 2.5 mmHg ± standard deviations derived by polarographic needle measurements for 5 different time points (T1 to T5) in RIF1 tumors. Without photosensitizer and without light, i.e. tracks only. Light only, illumination without photosensitizer and PDT; illuminations with photosensitizer: before (T1), during (T2 = 2 min, T3 = 11 min) and after (T4 = + 5 min, T5 = + 60 min) illumination.
Figure 2
Hematoxylin-eosin staining of RIF1 tumor (A) without treatment, no evidence of necrosis is seen and H-MESO1 tumor (B) with extensive formation of necrosis (arrows).

PDT treated groups as mentioned above (Table 3).

Untreated H-MESO1 tumors showed evidence of hypoxia in 0.6% and 3.0% of the tumor excluding necrotic areas at 22 and 82 minutes after injection of EF3 respectively (Figure 4, open bars). PDT induced significant increases in the extent of hypoxia only in the non necrotic tumor areas (Figure 5). Inclusion of necrotic areas in the calculation of hypoxic fractions did not make any difference in significance of relative changes between controls and PDT treated tumors (Table 3).

DISCUSSION

This study addressed possible oxygen depletion during and after photodynamic therapy in two different experimental tumor models. During illumination oxygen can become depleted due to insufficient delivery to the treated tissues to replenish consumption by the photochemical reaction. Oxygen depletion during PDT is reversible once illumination stops if it is caused by an imbalance between consumption and supply. PDT induced vascular damage can also occur during PDT and, if this results in the formation of fibrin emboli, this may lead to irreversible tissue hypoxia (25-28).
### Table 2. Oxygenation of Mesothelioma xenograft (H-MESO1) tumors in relation to PDT

Mean median pO2 in mm Hg ± STD

<table>
<thead>
<tr>
<th>Time point</th>
<th>Control tracks only</th>
<th>PDT tracks only</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>8.5 ± 6.0</td>
<td>6.2 ± 5.1</td>
</tr>
<tr>
<td>T2 illumination</td>
<td>10.1 ± 9.8</td>
<td>8.3 ± 9.4</td>
</tr>
<tr>
<td>T3 illumination</td>
<td>9.7 ± 8.9</td>
<td>8.6 ± 7.0</td>
</tr>
<tr>
<td>T4</td>
<td>10.4 ± 6.2</td>
<td>8.5 ± 4.6</td>
</tr>
<tr>
<td>T5</td>
<td>9.8 ± 7.1</td>
<td>7.4 ± 7.0</td>
</tr>
</tbody>
</table>

% negative values
- Control tracks only: 1.1
- PDT tracks only: 3.4

Most negative value
- Control tracks only: -1.9
- PDT tracks only: -5.3

Mice per group
- Control tracks only: 12
- PDT tracks only: 12

**Repeated Measurements – ANOVA**

<table>
<thead>
<tr>
<th>Slope ± SE</th>
<th>0.034 ± 0.057</th>
<th>0.073 ± 0.089</th>
</tr>
</thead>
<tbody>
<tr>
<td>p value slope</td>
<td>0.57</td>
<td>0.42</td>
</tr>
</tbody>
</table>

**Difference in slope between groups (p value)**

<table>
<thead>
<tr>
<th>PDT vs Tracks only</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.71</td>
</tr>
</tbody>
</table>

T1 = 5 minutes before start of illumination, T2 and 3 = 2 and 11 minutes after start of illumination, T4 and 5 = 5 and 60 minutes after end of illumination. In tracks only group: T1-T5, 5 minutes between measurements.
The RIF1 tumor was chosen for oxygenation measurement experiments, because it is known to be well vascularized without extensive formation of necrosis. Oxygen depletion has been demonstrated with Photofrin mediated interstitial (17,20) and superficial (18,29) PDT in this tumor model. The human mesothelioma xenograft H-MESO1 was studied as a model for possible oxygen depletion during clinical PDT after surgery in patients with malignant pleural mesothelioma (10).

Tumor tissue oxygenation was measured with an Eppendorf polarographic histograph, which is the most extensively tested direct method of measuring tissue oxygenation. Eppendorf measurements made in human tumors have shown a consistent negative correlation between tumor tissue oxygenation and response to both radiotherapy and surgery (30,31).

Our measurements in RIF1 tumors revealed a large spread of $pO_2$ values among untreated tumors. Previous studies have also shown large intra- and inter tumor variabilities with this technique (32,33). On average, the present study demonstrated a significant trend for a decrease in intratumor partial oxygen tension during illumination of photosensitized RIF1 tumors which continued until one hour after PDT. Sitnik et al previously demonstrated a decreased $pO_2$ of RIF1 tumors during Photofrin mediated PDT; this was already evident within 1 minute after starting illumination (18). Our experiments revealed a more gradual decrease in $pO_2$ during and after mTHPC mediated PDT. The mean median $pO_2$ of RIF1 tumors, measured one hour after illumination (T5) was 51% of the pretreatment oxygenation level (T1). This is slightly less than the 70% decrease in $pO_2$ reported in Sitniks study. In both studies a total light dose of 100 J.cm$^{-2}$ was used. The kind of photosensitizer used may influence the extent to which $pO_2$ decreases during PDT. Although mTHPC PDT has been demonstrated to be more effective than Photofrin PDT in comparable treatment schedules (34,35), this increased PDT efficacy does not necessarily lead to more severe tissue hypoxia. Efficacy of PDT may be related to other factors than only the total amount of singlet oxygen and oxygen radicals produced. Different photosensitizers can, for example, exhibit differences in local sensitization distribution and site of damage, which can also influence efficacy.

When baseline $pO_2$ levels in groups of mice exposed to mTHPC alone (PDT group T1) were compared with those without mTHPC (tracks only and light only T1), there was a non significant improvement in oxygenation after injection of only mTHPC. However, differences in $pO_2$ values between different experimental groups should be judged with caution. Significant variation in baseline values between experimental groups was previously reported by several authors (29,36). This should support the contention that reliable data on oxygenation changes can only be obtained within one experimental group.

Polarographic measurements made in H-MESO1 xenografts during and after PDT did not demonstrate any significant change in median $pO_2$ or the percentage values below 2.5 mmHg. The lack of measurable changes in $pO_2$ may be partly due to the extensive necrosis seen in this tumor. Relatively poor vascularization in necrotic parts of the H-MESO1 tumor may result in less striking differences in tumor tissue $pO_2$ during PDT.

The perforation made by each measuren track of the Eppendorf needle inevitably leads to some damage of tumor tissue including its vasculature. Theoretically this should lead to impairment of blood supply resulting in decreasing levels of oxygenation. However, such a decrease was not demonstrable in previous studies (37) and was also not observed in our experiments. The tracks only groups showed a slight, but consistent increase (RIF1) or no change at all (H-MESO1) of median $pO_2$ values during five consecutive measurement periods. Bleeding along the probe track (38) or recruitment of
Figure 3
Relative hypoxic fractions as measured by percentages EF3 binding + standard deviations in R1F1 tumors. Data are shown 22 and 82 minutes after EF3 injection, for tumors in mice injected with 0.15 mg.kg⁻¹ mTHPC 24 hours before analysis with (N = 3) or without (N = 3) illumination.

Figure 4
Relative hypoxic fractions as measured by percentages EF3 binding + standard deviations in H-MESO1 tumors. Data are shown 22 and 82 minutes after EF3 injection for tumors in mice injected with 0.15 mg.kg⁻¹ mTHPC 24 hours before analysis with (N = 3) or without (N = 3) illumination.
## Table 3. Hypoxic fraction in RIF1 and H-MESO1 tumours

<table>
<thead>
<tr>
<th>Time after EF3 injection</th>
<th>RIF1</th>
<th>H-MESO1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Necrosis</td>
<td>Necrosis</td>
</tr>
<tr>
<td>excluded</td>
<td>included</td>
<td>excluded</td>
</tr>
<tr>
<td>22'</td>
<td>0.5 ± 0.5</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td>22' with PDT</td>
<td>2.4 ± 1.4</td>
<td>2.3 ± 1.4</td>
</tr>
<tr>
<td>82'</td>
<td>1.0 ± 0.4</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>82' with PDT</td>
<td>14.3 ± 5.5</td>
<td>10.7 ± 4.5</td>
</tr>
</tbody>
</table>

Blood flow to mechanically damaged areas may possibly have interfered with the PDT induced changes in oxygen levels. However, the observed decrease in mean median pO₂ and increase in the percentage of pO₂ measurements < 2.5 mmHg can not be explained by the mechanical damage due to the insertion of the Eppendorf needle. In fact the decrease in oxygenation during PDT was even more pronounced when each measurement period of the PDT treated group was compared with the corresponding period of the tracks only group in RIF1 tumors.

Immunohistochemical staining was used as another method to investigate changes in tumor oxygenation in our two tumor models. Hypoxia markers have the advantage that information can be obtained about the spatial distribution of hypoxia within a tumor. However, bioreduction of hypoxia markers is time dependent and the rate of bioreduction depends on type of drug as well as oxygenation levels. EF3 was chosen for the present study, because bioreduction for this marker begins within 2 minutes (21), which allowed us to compare changes in oxygenation during and after PDT. The increase in EF3 staining with time observed in control tumors indicated that the bioreduction was however, not maximal at the earliest time point tested (22' after injecting EF3). The four-fold increase in staining of untreated tumor sections seen over the time period 22 to 82 minutes, correlates very well with the study of Busch et al which demonstrated a four-fold increase in EF3 binding in vitro when RIF1 cells were exposed to hypoxia over a for 60 minutes period (21). This indicates the necessity of comparing control and tested tumors at equivalent EF3 exposure times.
Figure 5
Matched images of H&E staining and EF3 labeled hypoxic areas for H-MESO1 tumors before (A) and 1 hour after (B) PDT. Hypoxia demonstrated by EF3 is not seen in areas of necrosis.

Half maximum effect of Photofrin mediated PDT occurs at 0.5-1% O₂ (24,39). For PDT using mTHPC this K value has not been established but it is likely to be in the same range. Bioreduction of EF3 is most effective between 0.1 and 1% oxygen (21). This drug therefore seems to be suitable for detection of areas with levels of hypoxia relevant for PDT. In spite of the fact that the most effective range of EF3 bioreduction occurs under conditions with a pO₂ lower than 2.5 mmHg, there was no direct correlation between the percentage of tumor area calculated to be hypoxic using EF3 staining and the percentage of Eppendorf pO₂ values < 2.5 mmHg. EF3 binding estimates of hypoxic fractions were much lower than the percentage of pO₂ values < 2.5 mmHg, particularly for control tumors. This may indicate that EF3 bioreduction, although starting within a few minutes after administration still continues for more than 82 minutes.

Experiments with the hypoxia marker EF3 indicated significant increases in hypoxia of both RIF1 and H-MESO1 after PDT. The 22 minute time point, directly after PDT illumination was chosen for comparison with T3 of the Eppendorf experiments, at the end of the illumination period. Results of EF3 staining obtained 82 minutes after EF3 injections were used for comparison with pO₂ values 60’ after the end of illumination (T5). EF3 experiments revealed a consistent rise in hypoxia of both tumors during and after PDT. Polarographic needle measurements only indicated a modest increase in
percentages of pO\textsubscript{2} values < 2.5 mmHg for RIF1 tumors. Lack of vascularization in necrotic parts of H-MESO1 tumors may have introduced artefacts in the pO\textsubscript{2} measurements. Theoretically polarographic needle measurements should detect hypoxia in necrotic regions, whereas such regions are not stained by a hypoxia marker because active metabolism is required for the bioreduction to occur. After correction for necrosis, EF3 staining demonstrated increased hypoxia during and after PDT for both RIF1 and H-MESO1 tumors. Correction for necrosis is impossible for Eppendorf experiments, unfortunately. Absence of correlation between tissue oxygenation results obtained with various methods was also found in two other studies (36,40). Eppendorf measurements did not correlate with either [3H] misonidazole binding nor with the hypoxic fraction as determined by survival assays. In contrast with these results, a recent publication demonstrated significant correlation between pimonidazole binding and the percentage of low pO\textsubscript{2}. Interestingly, mean median pO\textsubscript{2} values did not correlate with hypoxia marker data (41), which is supported by the H-MESO1 results in this study.

Recently we reported on polarographic measurements in H-MESO1 tumors after administration of nicotinamide and carbogen (42). Each of these additives individually caused an increase in mean median pO\textsubscript{2} and led to a significant improvement in PDT response. These data confirmed the role of oxygen as a critical factor for effective mTHPC mediated PDT in this tumor, despite the lack of evidence for a decrease in median pO\textsubscript{2} during PDT from the Eppendorf measurements. This suggests that EF3 measurements give a more relevant indication of the extent of PDT induced hypoxia in H-MESO1 tumors.

In summary, this study indicates oxygen depletion during and after mTHPC mediated PDT in both RIF1 and H-MESO1 tumors. Increase of tissue hypoxia was demonstrated in both tumors with the hypoxia marker EF3. Polarographic needle measurements only revealed a significant decrease in median tumor pO\textsubscript{2}, or increase in the percentage of low pO\textsubscript{2} values in the RIF1 tumor and not in the H-MESO1 tumor. Adequate tumor oxygenation should be considered a critical factor for effective PDT.

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