Erythropoietin Restores the Antitumor Effectiveness of Photodynamic Therapy in Mice with Chemotherapy-induced Anemia

Jakub Golestani, Dominika Olszewska, Pawel Mróz, Katarzyna Kozar, Rafał Kamiński, Ahmad Jalili, and Marek Jakóbiński

Department of Immunology, Center of Biostructure, The Medical University of Warsaw, 02-004 Warsaw, Poland

ABSTRACT

Purpose: The study was designed to examine the impact of anemia on the antitumor efficacy of photodynamic therapy (PDT) in a murine colon-26 adenocarcinoma model syngeneic with BALB/c mice.

Experimental Design: Acute hemolytic anemia was induced by a single i.p. injection of phenylhydrazine hydrochloride (150 mg/kg). Anemia induced by i.p. administration of carboplatin (100 mg/kg) was corrected by s.c. treatment with recombinant human erythropoietin (1000 units/kg/day). The effectiveness of PDT (10 mg/kg Photofrin, 150 J/cm² laser dose) was evaluated by measurements of the footpad edema and tumor volume. All of the RBC-related parameters were measured from the tail vein.

Results: Phenylhydrazine hydrochloride injection resulted in a blunted response of normal tissues to Photofrin-mediated PDT-induced edema formation. Similarly, the antitumor response in mice with hemolytic anemia was nearly completely abrogated. The antitumor effectiveness of PDT was also significantly diminished in a more realistic clinical situation when anemia was induced by administration of carboplatin. Importantly, administration of recombinant human erythropoietin completely restored the sensitivity of the tumor to PDT in carboplatin-treated mice.

Conclusions: These results indicate that anemia can negatively influence the therapeutic effectiveness of PDT. For optimal antitumor response anemia should be corrected before PDT procedure.

INTRODUCTION

Anemia is a frequent complication of cancer occurring in up to 60% of patients (1). It might result from the malignant disease itself, accompanying infections or from chemotherapy administered to cancer patients (2). Regardless of the cause, anemia in cancer patients has a complex and generally negative impact on the disease. The complications of anemia result from hypoxia of virtually all organs. Cancer-associated anemia can severely affect the quality of life contributing to inability to work, depression, fatigue, and unsuccessful social life (3). Moreover, patients with anemia have a poorer outcome of medical interventions including radiotherapy and chemotherapy (4–6).

Several observations indicate that hypoxia might also influence the antitumor effectiveness of PDT. PDT involves the combination of visible light and a photosensitizer (7). Neither of the PDT components alone can induce antitumor effects, but when combined with oxygen they produce lethal cytotoxic agents that can either directly kill tumor cells or destroy blood vessels within the tumor, thus contributing to the antitumor effects (7). Because reactive oxygen species generated during PDT arise from the ground state oxygen (8) it is apparent that oxygen availability is a rate-limiting factor influencing the effectiveness of treatment. Early observations indicate that hypoxic or anoxic conditions almost completely reduce the antitumor effectiveness of PDT in vitro (9). Several mechanisms could limit oxygenation of tumor tissue undergoing PDT: photochemical oxygen consumption during the photodynamic process itself (10, 11); destruction of microvessels within the tumor (12, 13); and increased coagulation with accompanying decrease in blood flow (14). Therefore, the damaging effects of PDT on the microvasculature are diminishing oxygen supply. Indeed, PDT was shown to be ineffective in poorly vascularized xenograft model (15), and mathematical modeling shows that the rate of oxygen consumption during PDT is sufficient to drive tumor tissue into very low levels of oxygenation making the rate of oxygen diffusion from capillaries ineffective (16). With some photosensitizers, such as Photofrin, these effects can be sufficient to drive fractions of the tumor into such low oxygen concentration that the efficacy of PDT is to some extent limited (7).

Thus, we decided to investigate whether the influence of low blood oxygen carrying capacity resulting from tumor accompanying anemia might be a factor that a priori makes PDT less effective than in normocytic conditions. Moreover, if this would be the case we wanted to check whether erythropoietin...
can restore the effectiveness of PDT in anemic tumor-bearing mice.

MATERIALS AND METHODS

Mice and Tumor. BALB/c mice, 8–12 weeks of age, were used in the experiments. Breeding pairs were obtained from the Institute of Oncology (Warsaw, Poland). All of the experiments with animals were performed in accordance with the guidelines approved by the Ethical Committee of the Medical University of Warsaw. Poorly differentiated colon adenocarcinoma cells, C-26, were used throughout the experiments. Cells were cultured in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% heat-inactivated FCS, antibiotics, 2-mercaptoethanol (50 μM), and L-glutamine (2 mM; all from Life Technologies, Inc.). For in vivo experiments exponentially growing tumor cells were harvested, resuspended in PBS medium to appropriate concentration of cells, and injected (1 × 10⁶ C-26 cells in 20 μl PBS) into the footpad of the right hind limb of experimental mice. Tumor cell viability (measured by trypan blue exclusion assay) ranged between 95 and 98%.

Reagents. Photofrin, was a generous gift of QLT Phototherapeutics, Inc. (Vancouver, British Columbia, Canada). It was diluted with 5% dextrose before i.p. administration. rHuEpo (Epoetinum B) was purchased from Roche (Basel, Switzerland). Erythropoietin was diluted with 0.9% NaCl immediately before s.c. administration. PH was purchased from Sigma Chemical Co.

PH- and Carboplatin-induced Anemia. Acute hemolytic anemia was induced by i.p. injection of freshly prepared PH. PH was dissolved in PBS, the pH was adjusted to pH 7.4 with NaOH, and injected at three different doses of 50, 100, and 150 mg/kg. Prolonged anemia was induced by a single i.p. injection of carboplatin (Polfa) at a dose of 100 mg/kg dissolved in 0.9% NaCl (see also Fig. 3). Mice in control groups were injected with 0.9% NaCl. Blood was collected from tail vein, and peripheral blood cells were assessed using a Sysmex-820 cell counter (Sysmex, Kyoto, Japan) adapted for the analysis of rodent cells. All of the experiments were performed with tumor-bearing mice.

Erythropoietin Treatment. Erythropoietin was administered s.c. at a dose of 1000 units/kg/day. The first dose was given 10 days before the inoculation of C-26 cells, and the treatment continued for 17 consecutive days (see also Fig. 3).

Photodynamic Tumor Treatment and Monitoring of Tumor Growth. Photodynamic tumor treatment was done essentially as described earlier (17). Briefly, Photofrin was administered i.p. at a dose of 10 mg/kg, 24 h before illumination with 630 nm of light on day 6 after inoculation of tumor cells (controls received 5% dextrose). The light source was a He-Ne ion laser (Laserinstruments, Warsaw, Poland). The light was delivered on day 7 of the experiment using a fiberoptic light delivery system. The power density at the illumination area, which encompassed the tumor and 1–1.5 mm of the surrounding skin, was ~80 mW/cm² (40 mW laser output). The total light dose delivered to the tumors was 150 J/cm². During the light treatment mice were anesthetized with ketamine (87 mg/kg) and xylazine (13 mg/kg), and restrained in a specially designed holder. Local tumor growth was determined as described (18) by the formula:

Relative tumor volume = [(tumor volume) / (initial tumor volume)] × 100%. The initial tumor volume ranged from 18 to 22 mm³.

Statistical Analysis. Data are presented as means ± SD. Differences in tumor volume, footpad diameter, and hematological parameters were analyzed for significance by Student’s t-test. Additionally, data from in vivo studies were analyzed with the nonparametric Mann-Whitney U test (Instat; GraphPad Software, San Diego, CA). Significance was defined as a two-sided P < 0.01.

RESULTS

In the initial experiments we have established PH doses that induced acute and severe hemolytic anemia. Tumor-bearing mice were i.p. injected with a single dose of 50, 100, or 150 mg/kg of PH, and were daily monitored for RBC count and hemoglobin concentration in peripheral blood. As shown in Table 1, PH induced a dose- and time-dependent anemia that reached its nadir on day 3 and was the most severe in a group receiving 150 mg/kg. This dose was used in additional experiments.

PDT is usually accompanied by tissue edema at the site of laser illumination. Therefore, we have determined the influence of PDT on the edema formation in the footpads of nonanemic
control animals and in mice injected with 150 mg/kg of PH 3 days before laser illumination. As shown in Fig. 1, PDT induced a significant tissue thickening in nonanemic animals (423% ± 76% increase in footpad volume). Of note, there was a significantly reduced footpad thickening in anemic animals (205% ± 28% increase in footpad volume) indicating that low blood oxygen carrying capacity might reduce the photodynamic effects in tissues (P < 0.01; Student’s t test).

Next, we have decided to compare the antitumor effectiveness of PDT in nonanemic controls versus animals with PH-induced anemia. Tumor-bearing mice were injected with either PBS or PH (150 mg/kg) on day 4 after inoculation of C-26 cells. Two days later mice received either Photofrin (10 mg/kg) or 5% dextrose as a control. After another 24 h, Photofrin-inoculated mice were illuminated with laser light. PDT induced a significant inhibition of tumor growth (Fig. 2). Remarkably, the PDT in anemic animals was not effective.

To investigate the antitumor effectiveness of PDT in a more realistic clinical situation we have adapted a model of chemotherapy-induced anemia used by Thews et al. (Ref. 19; Fig. 3A). Carboplatin (100 mg/kg) administration 4 days before tumor cell inoculation induced a moderate anemia that persisted for at least 11 days (data not shown). We then investigated the antitumor effectiveness of PDT in mice with carboplatin-induced anemia and compared it with effectiveness in nonanemic animals as well as in mice treated with rHuEpo. The tumor growth curves in the nonanemic control group as well as in carboplatin-alone treated (anemic) and rHuEpo-treated mice were comparable (Fig. 3B). Similarly, the growth of tumors treated with PDT in nonanemic and rHuEpo-treated mice was indifferent (Fig. 3B). These results indicate that neither carboplatin-induced anemia (4 days before inoculation of tumor cells) nor rHuEpo treatment had any impact on the growth rate of the investigated tumors. These data are comparable with the results obtained by Thews et al. (19). Importantly, PDT produced a significant retardation of tumor growth. The effectiveness of PDT in carboplatin-treated (anemic) animals was significantly decreased, but remarkably rHuEpo treatment completely restored the antitumor effects of photodynamic treatment (Fig. 3C). This experiment was accompanied by the measurements of RBC-related parameters in age- and sex-matched animals. Table 1 shows that on the day of laser illumination (day 11 after carboplatin administration) there was a moderate anemia in carboplatin-treated mice, which was completely corrected by rHuEpo administration (Table 2).

DISCUSSION

Tumor hypoxia is frequently considered a potential therapeutic problem because it renders solid tumors more resistant to ionizing radiation and may also confer decreased sensitivity to most anticancer drugs (20). Continued hypoxia may result in cellular changes leading to a more aggressive tumor phenotype as reflected by accelerated malignant progression, increased potential for local invasiveness, and tumor cell spreading (21).

Several different approaches have addressed the problem of excessive oxygen consumption during PDT. Reduction of the fluence rate was shown to improve the efficacy of PDT by augmenting tumor oxygenation (11, 22, 23). Similarly, several studies indicated that the use of fractionated light delivery with either short- or long-term intervals significantly improves the efficacy of PDT by allowing reoxygenation of tumor tissue during dark periods (24, 25). In other studies it was demonstrated that hyperbaric oxygen can enhance the effects of PDT.
All of these observations indicate that appropriate oxygen delivery is among the critical parameters influencing the successful tumor response after PDT (24, 28).

In the present studies we tried to establish the influence of anemia on the effectiveness of PDT. PH is a frequently used agent that induces a rapid and severe hemolytic anemia in experimental animals (29). As shown in Fig. 1 the induction of acute and severe hemolytic anemia by administration of PH is significantly diminishing the tissue edema that usually accompanies PDT effects. These results indicate that in the anemic animals the photodynamic reaction is somewhat impaired and might also result in decreased antitumor effects of PDT. Indeed, experimental animals (29). As shown in Fig. 1 the induction of acute and severe hemolytic anemia by administration of PH is significantly diminishing the tissue edema that usually accompanies PDT effects. These results indicate that in the anemic animals the photodynamic reaction is somewhat impaired and might also result in decreased antitumor effects of PDT. Indeed,

---

**Table 2** Erythropoietin restores carboplatin-induced anemia on the day of laser illumination

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Carboplatin (100 mg/kg)</th>
<th>rHuEpo (1000 units/kg/day)</th>
<th>Carboplatin + rHuEpo</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC count [\times 10^{12}/liter]</td>
<td>11.52 ± 1.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.02 ± 1.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.82 ± 1.55</td>
<td>12.06 ± 0.96&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>HGB [g/liter]</td>
<td>133.83 ± 23.40</td>
<td>76.67 ± 16.46&lt;sup&gt;d&lt;/sup&gt;</td>
<td>174.33 ± 25.11</td>
<td>140.17 ± 9.02&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hematocrit [%]</td>
<td>57.33 ± 9.00</td>
<td>35.43 ± 4.95&lt;sup&gt;d&lt;/sup&gt;</td>
<td>79.55 ± 9.10</td>
<td>61.25 ± 8.53&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Balb/c mice \(n = 6\) were treated according to the schedule shown in Fig. 3A. On day 7 after inoculation of C-26 cells four groups (controls, carboplatin, rHuEpo, and carboplatin + rHuEpo) were split into two experiments. Some mice \(n = 7–9\) were used in the experiments evaluating the antitumor effectiveness of PDT in normal and anemic animals (data are presented in Fig. 3, B and C), and the remaining six animals from each group were used for the analysis of RBC-related parameters.

<sup>b</sup> Data represent means ± SD.

<sup>c</sup> \(P < 0.01\) (Student’s \(t\)-test) vs. controls.

<sup>d</sup> \(P < 0.01\) (Student’s \(t\)-test) vs. carboplatin-treated mice.

---

Fig. 3 Antitumor effects of the PDT in mice with carboplatin-induced anemia. A, the treatment with rHuEpo (1000 units/day s.c.) was started on day 10 of the experiment (day 0 is the day of tumor cells inoculation). On day 4 mice were injected with carboplatin (100 mg/kg). On day 6 mice were injected with Photofrin (10 mg/kg), and laser illumination (120 J/cm²) was done on day 7. All necessary controls were included where necessary (0.9% NaCl as a control for rHuEpo and carboplatin injections, 5% dextrose as a control for Photofrin). B, the influence of carboplatin, rHuEpo, and the combinations of PDT + rHuEpo and carboplatin + rHuEpo on the growth of C-26 tumors in BALB/c mice \(n = 7–9\). \(P < 0.01\) (Mann-Whitney \(U\) test): PDT + Epo-treated mice in comparison with all other groups. C, the influence of PDT and the combinations of carboplatin + PDT and carboplatin + rHuEpo + PDT on the growth of C-26 tumors in BALB/c mice \(n = 7–9\). The data presented in B and C represent results from the same experiment, and the control group on both graphs is identical. Measurements of tumor diameter started on day 7 after inoculation of tumor cells. The data represent relative tumor volume (% of the initial tumor volumes on day 7) ± 95% confidence intervals. \(P < 0.01\) (Mann-Whitney \(U\) test): carboplatin + PDT-treated mice in comparison with controls. \(P < 0.01\) (Mann-Whitney \(U\) test): groups of mice treated with PDT or PDT in combination with rHuEpo and with carboplatin in comparison with all other groups.
as demonstrated in Fig. 2 the antitumor effectiveness of PDT was nearly completely abolished in PH-treated mice. The results of these studies unequivocally demonstrate that anemia significantly decreases the antitumor effectiveness of PDT in mice.

In the next series of experiments we evaluated the effects of PDT in mice with chemotherapy-induced anemia. As a chemotherapeutic we chose carboplatin, because it induces a normocytic, normochromic anemia persisting for at least 11 days (30). This long-lasting anemia allowed us to disregard any influence of carboplatin on the antitumor effects of PDT itself (several chemotherapeutics were shown previously to influence the effectiveness of PDT when administered at the same time; Refs. 31, 32). The effectiveness of PDT in carboplatin-treated (anemic) animals was significantly reduced. We were also interested in whether administration of rHuEpo, which is capable of preventing carboplatin-induced anemia, could restore the antitumor effects of PDT. Because carboplatin-induced anemia is presumed to result from myelosuppression, we began rHuEpo treatment before administration of the chemotherapeutic. Remarkably, rHuEpo completely restored the PDT effectiveness in carboplatin-treated mice.

Anemia correlates with a worsening of the tumor oxygenation status (33). The mechanisms of reduced effectiveness of PDT in the setting of hypoxia are probably multifactorial and most probably result from poor oxygen supply. However, other mechanisms such as decreased photosensitizer uptake cannot be excluded. Because PDT consumes enormous amounts of oxygen, it is possible that the oxygen reserves in hypoxic tumors of anemic animals are exhausted much sooner resulting in a decreased antitumor efficacy. rHuEpo could restore the oxygenation level before PDT thus optimizing the effectiveness of the treatment.

Altogether, the results of these studies indicate for the first time that anemia may cause at least partial nonresponsiveness of tumors to PDT. Importantly, rHuEpo is capable of restoring the sensitivity of tumors to PDT. Treatment with rHuEpo is not warranted in combination with PDT in nonanemic individuals. These studies are of immediate clinical application.

REFERENCES


Erythropoietin Restores the Antitumor Effectiveness of Photodynamic Therapy in Mice with Chemotherapy-induced Anemia

Jakub Golab, Dominika Olszewska, Pawel Mróz, et al.