Chapter 36
Tumor Reoxygenation Following Administration of the EGFR Inhibitor, Gefitinib, in Experimental Tumors

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Abstract It is well recognized that tumor hypoxia is a critical determinant for response to therapy. The effect of an EGFR inhibitor/gefitinib (Iressa®) on tumor oxygenation was monitored daily using in vivo EPR (electron paramagnetic resonance) oximetry on TLT and FSaII tumor models. An increase in pO_2 was shown at a dose of 45 mg/kg i.p. ($n = 4$/group/tumor model). This allowed the identification of a window of reoxygenation in both tumor models (with a maximum between 15 and 20 mmHg after 2 days of treatment). The increase in tumor oxygenation was shown to be the result of a decrease in oxygen consumption. This is the first report on the effect of gefitinib on oxygen consumption by tumor cells and subsequent increase in tumor oxygenation in vivo.

36.1 Introduction

Over the last several years, clinical studies have shown that hypoxia is an independent prognostic indicator of poor patient survival in different tumor types [1]. The oxygenation status of a tumor has been shown to be a pivotal factor in the efficacy of standard radiotherapy [2] because of the so-called oxygen enhancement effect, and the radiation dose required to achieve the same biological effect is about three times higher in the absence of oxygen than in the presence of normal levels of oxygen [3, 4]. The hypoxic microenvironment causes a reduced formation of radiation-induced

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damaging free radicals that fixes damages to DNA, and thus, the overall therapeutic response to radiotherapy is compromised and reduced [5]. A particular area under focus is therefore to combine radiotherapy with co-treatments that are able to transiently increase tumor oxygenation at the time of irradiation. Gefitinib, described as an EGFR inhibitor, has been shown to be able to reduce tumor hypoxia in experimental models [6]. Measurements of hypoxia have been done using in vivo small animal positron emission tomography (PET) imaging with the hypoxia marker [18F] fluoroazomycin arabinoside (FAZA). Gefitinib has also been shown in independent studies to potentiate the effect of radiation therapy [7]. Gefitinib (Iressa or ZD1839) is an orally active, reversible inhibitor of the EGFR tyrosine kinase [8]. In the EU, gefitinib (Iressa) is indicated for the treatment of adult patients with locally advanced or metastatic non-small cell lung cancer (NSCLC). In the current study, we monitored the effect of gefitinib longitudinally on tumor oxygenation, using in vivo electron paramagnetic resonance oximetry, on two distinct experimental tumor models. After identification of a window of reoxygenation by daily measurements of FSaII and TLT tumor pO_2, we investigated the underlying mechanisms responsible for the reoxygenation effect.

36.2 Material and Methods

36.2.1 Animal, Tumor Models, and Treatments

A transplantable liver tumor model (TLT) [9] and a fibrosarcoma (FSaII) [10] were inoculated in the leg of NMRI and C3H/HeOuJIco mice, respectively. For inoculation, approximately 10^6 cells in 0.1 ml of media were injected intramuscularly into the right rear leg. Tumors were allowed to grow up to 8 mm in diameter prior to experimentation. All animal experiments were conducted in accordance with national and university animal care regulations. Gefitinib was purchased from LC Laboratories (MA USA) and was dissolved in DMSO (Invitrogen) (final concentration: 11.25 mg/ml) and delivered daily i.p. at a dose of 45 mg/kg body weight. Control animals were injected with DMSO only.

36.2.2 In Vivo Tumor pO_2 Measurement

In vivo tumor pO_2 was monitored daily using EPR oximetry. The technique relies on the oxygen-dependent broadening of the EPR line width of a paramagnetic oxygen sensor implanted in the tumor [11, 12]. The localized EPR measurements obtained with the 1.2 GHz (or L band) spectrometer correspond to an average of pO_2 values in a volume of 10 mm^3. Data acquisition was performed every day before the injection of gefitinib or vehicle during 4 days, on both TLT and FSaII tumor models (45 mg/kg, $n=4$ for TLT and FSaII); control group received daily DMSO injections ($n=4$ for TLT and FSaII).

36.2.3 Tumor Blood Flow Estimation

Patent blue (Sigma-Aldrich, Belgium) staining was used to obtain a rough estimate of FSaII tumor perfusion [13] at day 2 after treatment with gefitinib ($n=6$) or vehicle (DMSO, $n=14$). This technique involves the injection of 200 µL of a Patent Blue solution (1.25 %) into the tail vein of mice. After 1 min, a uniform distribution of the staining through the body was obtained and mice were sacrificed. Tumors were carefully excised and cut into size-matched halves. Pictures of each tumor cross section were taken with a digital camera. To compare the stained versus unstained area, an in-house program running on Matlab (Mat work, Natick, MA, USA) was developed. The mean of the percentages of the two pictures was then calculated and was used as an indicator of tumor perfusion.

36.2.4 Ex Vivo Oxygen Consumption Rate Evaluation

The method described by James et al. [14] was used. All spectra were recorded on a Bruker EMX EPR spectrometers operating at 9 GHz. FSaII tumor-bearing mice were treated for 2 days with gefitinib as described above ($n=4$ for gefitinib and $n=3$ for vehicle). At day 2 after treatment, the mice were sacrificed and the tumor was excised. FSaII tumors were then dissected in a sterile environment and gently pieced in McCoy's medium. Cells were suspended in 10 % dextran in complete medium. The sealed tubes were placed into quartz EPR tubes, and samples were maintained at 37 °C. As the resulting line width reports on O_2 concentration, oxygen consumption rates were obtained by measuring the O_2 concentration in the closed tube over time and determining the slope of the resulting linear plot.

36.2.5 Statistical Analysis

Results are given as means ± SE values from n animals. Comparisons between groups were made with Student's t test or one-way ANOVA along with post hoc Dunnett's multiple comparison tests when appropriate. P values <0.05 (*), <0.01 (**), or <0.001 (***) were considered significant.

36.3 Results and Discussion

We observed an increase in pO_2 in both TLT and FSaII tumor models from day 1 until day 3 post treatment (this increase was not observed for the control group). The maximal pO_2 was reached after 2 days of treatment. This value was significantly higher than before treatment ($P<0.05$) (Figs. 36.1 and 36.2). We chose day 2 as the "window of reoxygenation" for the rest of the experiments. Since an increase

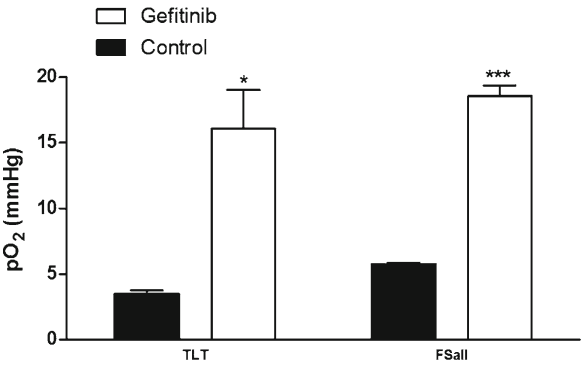


Fig. 36.1 TLT and FSaII tumor oxygenation after 2 days of treatment with gefitinib, assessed by EPR oximetry. Day 2 was selected as the window of reoxygenation for the rest of the experiments

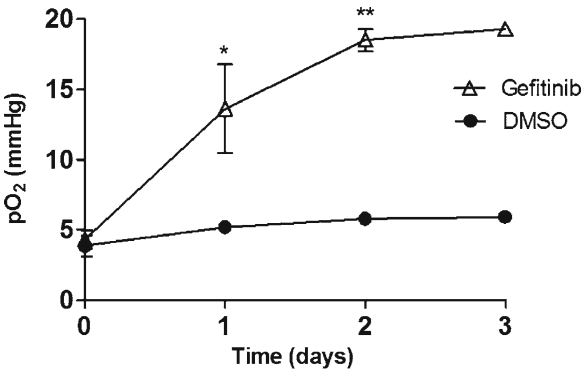


Fig. 36.2 Effect of daily injections of gefitinib (45 mg/kg, $n=4$) on FSaII tumor oxygenation, as monitored using EPR oximetry. Control group received daily DMSO (vehicle) injections ($n=4$). Note the significant increase in tumor oxygenation induced by gefitinib

in tumor oxygenation can be due to both an increase in oxygen supply (blood flow) and/or a decrease in oxygen consumption by tumor cells, both parameters were assessed in the following experiments.

The administration of the drug significantly decreased the oxygen consumption rate of FSaII cells, with a slope of 0.65 μM/min for the control group and 0.26 μM/min for gefitinib ($P<0.01$) (Figs. 36.3 and 36.4).

Here, the main factor that is likely to be responsible for the increase in tumor oxygenation is the decrease in the oxygen consumption rate by tumor cells. This is in accordance with a mathematical model that predicted that modification of oxygen consumption would be much more efficient at alleviating hypoxia than modification of oxygen delivery [15], a theoretical hypothesis that has been further illustrated experimentally in our laboratory using several agents able to target the oxygen consumption by tumor cells [16–18].

In this study, we observed a decrease in oxygen consumption after treatment with gefitinib that might be caused by mitochondrial impairment. Although this remains to be demonstrated in the tumor models used in this study, gefitinib was shown to cause significant mitochondrial membrane depolarization in different breast cancer cell lines [19]. These data suggest a role of the mitochondrial dysfunction as a potential factor at the origin of the decrease in oxygen consumption by tumor cells consecutive to treatment with gefitinib.

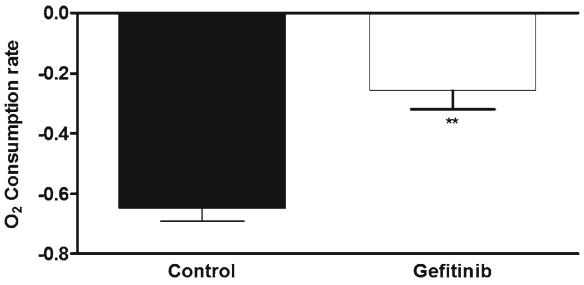
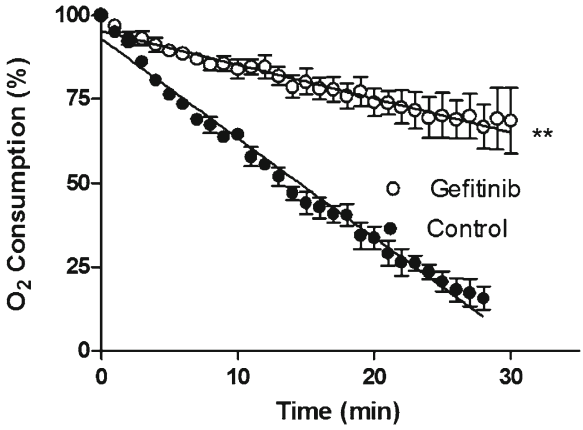


Fig. 36.3 Effect of gefitinib on the rate of oxygen consumption by FSaII tumor cells, as measured ex vivo after 2 days of treatment using EPR oximetry. Note the significant decrease in oxygen consumption rate in gefitinib group ($n=4$) compared to the control group ($n=3$) ($p<0.01$)

Fig. 36.4 Ex vivo tumor cells' oxygen consumption measurement using EPR oximetry after 2 days of treatment with gefitinib ($n=4$)



The second factor that is able to influence pO_2 is the modification of blood flow (supply) that regulates oxygen delivery to tumors. We measured the ratio (colored area/complete area) observed in tumors after injection of a blue dye. Tumors treated with gefitinib ($n=6$) showed a colored area of 17.4 ± 2.7 % and tumors treated with vehicle ($n=14$) showed a colored area of 11.2 ± 0.9 % (Fig. 36.5). We did not observe any significant modification in blood flow during treatment with gefitinib, contrarily to what we recently observed after administration of the MAPK and VEGFR inhibitor sorafenib [20]. Therefore, the major factor responsible for the increase in tumor oxygenation is the reduction in oxygen consumption by tumor cells.

These results might support the radiosensitizing effect of gefitinib combined with radiation described in other studies [21], where gefitinib was shown to radiosensitize glioma cells by mediated DNA double-strand break repair and enhanced irradiation-induced DNA damage [21]. Increased tumor oxygenation may explain enhancement of irradiation DNA damage [22]; indeed in the presence of oxygen, DNA damage can be stabilized through oxidation of DNA radicals [22].

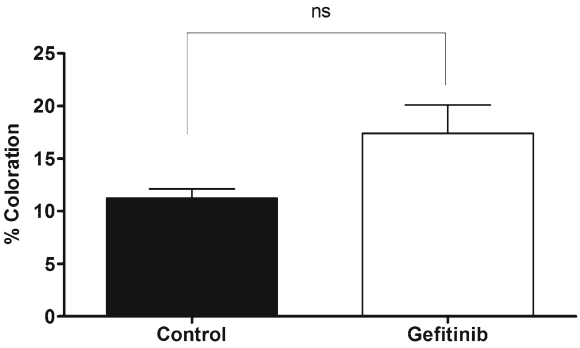


Fig. 36.5 Mean percentage of perfused areas in FSaII tumors assessed by patent blue staining after 2 days of treatment with gefitinib ($n=6$) and control ($n=14$)

To conclude, we observed an increase of pO_2 in TLT and FSaII tumors after 2 days of treatment with gefitinib. The effect is likely to be due to a major decrease in oxygen consumption by tumor cells. This study provides a rationale for using gefitinib as co-treatments for radiation therapy.

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