

Thyroid Status is a Key Modulator of Tumor Oxygenation: Implication for Radiation Therapy

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In normal tissues, thyroid hormones play a major role in the metabolic activity and oxygen consumption of cells. Because the rate of oxygen consumption is a key factor in the response of tumors to radiation, we hypothesized that thyroid hormones may affect the metabolic activity of tumor cells and hence modulate the response to cytotoxic treatments. We measured the influence of thyroid status on the tumor microenvironment in experimental tumors. Hypothyroidism and hyperthyroidism were generated in mice by chronic treatment with propyl thiouracil and L-thyroxine. Thyroid status significantly modified tumor pO_2 as measured with EPR oximetry. Mechanistically, this was the result of the profound changes in oxygen consumption rates. Thyroid status was associated with a significant change in tumor radiosensitivity since the regrowth delay was increased in hypothyroid mice compared to euthyroid mice, an effect that was abolished when temporarily clamped tumors were irradiated. This study provides unique insights into the impact of modulating tumor oxygen consumption and could have implications in the management of cancer patients with thyroid disorders. © 2007 by Radiation Research Society

INTRODUCTION

Thyroid hormones are required for normal function of nearly all tissues, with major effects on oxygen consumption and metabolic rate (1, 2). In tumors, there is a coherent body of evidence suggesting that thyroid hormones modulate multiple neoplasia-dependent mechanisms (3). The role of thyroid hormones in tumor growth has been investigated in multiple experimental models, including mammary carcinoma (4), fibrosarcoma (5), chondrosarcoma (6), colon

carcinoma,² hepatoma (7) and prostate cancer (8). These studies have demonstrated that hypothyroidism slows the neoplastic process, whereas administration of TH preparations restores tumor growth rates. Similarly, Gustavsson *et al.* (9) demonstrated a longer latent period before tumor development as well as a slower tumor growth rate in mice treated with PTU (propylthiouracil). In humans, several case reports have indicated that patients with cancer who have hypothyroidism may have a prolonged course compared with euthyroid patients, suggesting that the presence of hypothyroidism may be associated with prolonged survival, with the exception of patients with thyroid tumors (10). More recently, Cristofanilli *et al.* (11) showed that spontaneous clinical hypothyroidism may reduce the incidence of breast cancer and decrease its aggressiveness.

Davis *et al.* (12) recently showed that the TH L-thyroxine (T4) stimulates growth of cancer cells through a plasma membrane receptor on integrin $\alpha_v\beta_3$ in three glioma cell lines. These authors thus provided a cellular mechanism by which thyroid hormones can be a growth factor for glioma and a rationale for the clinical observations that reducing physiological levels of TH may increase the duration of survival in patients with glioblastoma.

Apart from direct action of thyroid hormones on tumor growth or incidence, it has been suggested that a decrease in thyroid function may serve to favorably influence the response to treatment. For example, animal experiments have demonstrated that low levels of circulating thyroid hormones may result in an increased response to chemotherapy. In a transplantable mouse mammary carcinoma model, a significant increase in complete responses was observed in hypothyroid mice treated with 5-fluorouracil (4). In clinical studies, hypothyroidism has been positively correlated with responses to treatment. For example, an enhanced response rate to cytotoxic chemotherapy has been observed in hypothyroid women with metastatic breast cancer (13). Similar results were observed in patients with metastatic renal cell carcinoma receiving cytokine-based ther-

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² I. Leith and A. Hercbergs, Effects of modulation of thyroid function on the growth and radiation sensitivity of a human colon carcinoma (HT29). Presented at the American Association for Cancer Research, 1994.

apy (3). Furthermore, patients treated for recurrent high-grade gliomas with high-dose tamoxifen survived significantly longer when chemical hypothyroidism was induced with PTU (14). Regarding radiotherapy, a retrospective study of 28 biochemically hypothyroid patients with inoperable solid tumors showed a complete plus partial response rate of 100% after a wide range of doses of radiation.³

Chronic alterations in thyroid status specifically affect mitochondrial oxygen consumption in skeletal muscle (15). To our knowledge, there is no comprehensive study of the effects of thyroid status on metabolism in tumor cells. This effect on tumor metabolism could be particularly relevant since it is well established that modulators of tumor oxygen consumption may have a dramatic effect on radiosensitivity (16–18). The aim of the present study was therefore to investigate the consequences of thyroid status (hypo-, hyper- or euthyroid) on tumor oxygen consumption as well as its effects on tumor oxygenation and on tumor regrowth delays after radiation therapy. This study provides major insights into the impact of the modulation of tumor metabolism and could have important implications in the management of cancer patients with thyroid disorders.

METHODS

Treatments and Tumor Models

NMRI mice were treated with PTU (propyl thiouracil) at 0.05% or L-thyroxine (0.003%) in drinking water. These concentrations have been shown to induce hypo- and hyperthyroidism in mice after 3 weeks of treatment (8). A transplantable liver tumor model (19) was then implanted in the legs of both the treated mice and the untreated (control) mice. For inoculation, approximately 10^6 cells in 0.1 ml of medium were injected intramuscularly into the right leg of NMRI mice. Mice developed palpable tumors within a week of inoculation. Tumors were allowed to grow to 8 mm in diameter prior to experimentation. Animals were anesthetized by inhalation of isoflurane mixed with 21% oxygen in a continuous flow (1.5 liter/h), delivered by a nose cone. Induction of anesthesia was done using 3% isoflurane. It was then stabilized at 1.8% for a minimum of 15 min before any measurement. The temperature of the animals was kept constant using IR light or a homeothermic blanket control unit. The animal facility is approved by the Belgian Ministry of Agriculture and the ethical comity (COMET) of the Catholic University of Louvain approved the protocols (protocol number 2004/UCL/MD028). All experiments were conducted according to national animal care regulations.

EPR Oximetry

EPR oximetry relies on the oxygen-dependent broadening of the EPR line width of a paramagnetic oxygen sensor implanted in the tumor (20). The technique is intended for continuous measurement of local pO_2 without altering the local oxygen concentration. EPR spectra were recorded using an EPR spectrometer (Magnetech, Berlin, Germany) with a low-frequency microwave bridge operating at 1.2 GHz and extended loop resonator. Charcoal (Charcoal wood powder, CX0670-1; EM Science, Gibbstown, NJ) was used as the oxygen-sensitive probe in all of the experiments (21). Calibration curves were made by measuring the EPR line width as a function of the pO_2 . For this purpose, the charcoal was

suspended in a tumor homogenate, and EPR spectra were obtained on a Bruker EMX EPR spectrometer (9 GHz) between 0 and 21% O_2 . Nitrogen and air were mixed in an Aalborg gas mixer (Monsey, NY), and the oxygen content was analyzed using a Servomex oxygen analyzer OA540 (Analytic Systems, Brussels). Mice were injected in the center of the tumor (6 mm diameter) using the suspension of charcoal (100 mg/ml, 50 μ l injected, 1–25 μ m particle size). EPR measurements were started 2 days after the injection. The tumor under study was placed in the center of the extended loop resonator, the sensitive volume of which extended 1 cm into the tumor mass, using a protocol described previously (20, 21). 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 mm³.

Evaluation of Oxygen Consumption Rate

All spectra for cell measurements were recorded on a Bruker EMX EPR spectrometer operating at 9 GHz. Tumors were excised and trypsinized for 30 min, and cell viability was determined. Cells (2×10^7 /ml) were suspended in 10% dextran in complete medium. A neutral nitroxide, ¹⁵N 4-oxo-2,2,6,6-tetramethylpiperidine-d₁₆-¹⁵N-1-oxyl at 0.2 mM in saline (CDN Isotopes, Pointe-Claire, Quebec, Canada), was added to 100- μ l aliquots of tumor cells that were then drawn into glass capillary tubes. The probe (0.2 mM in 20% dextran in complete medium) was calibrated at various O_2 levels between 100% nitrogen and air so that the line width measurements could be related to O_2 at any value. Nitrogen and air were mixed in an Aalborg gas mixer. The sealed tubes were placed into quartz ESR tubes, and samples were maintained at 37°C. Since the resulting line width reports on O_2 , oxygen consumption rates were obtained by measuring the O_2 in the closed tube over time and finding the slope of the resulting linear plot (17).

Tumor Regrowth Delay Assay

The tumor-bearing leg was irradiated locally with 10 Gy of 250 kV X rays (RT 250; Philips Medical Systems). Mice were anesthetized, and the tumor was centered in a 3-cm-diameter circular irradiation field. When tumors reached 8.0 ± 0.5 mm in diameter, the mice were randomly assigned to a treatment group and irradiated. After treatment, tumors were measured every day until they reached a diameter of 16 mm, at which time the mice were killed humanely. A linear fit to the tumor diameter could be obtained between 8 and 16 mm, which allowed us to determine the time to reach a particular size for each mouse. For each tumor, transverse and anteroposterior measurements were obtained. An average tumor diameter was then calculated. In a first set of experiments, three unirradiated groups of tumors (sham-treated, PTU-treated, L-thyroxine-treated) were compared with the three corresponding irradiated groups. In a second set of experiments including only irradiated groups, we compared PTU-treated tumors clamped during irradiation with sham-treated tumors + irradiation and PTU-treated tumors + irradiation. Tumor clamping was performed by ligation of the leg.

RESULTS

Effect of Thyroid Status on Tumor Oxygenation

PTU-treated mice (hypothyroid mice) showed a significantly higher level of tumor oxygenation than untreated mice; the mean tumor pO_2 was 11.5 ± 3.2 mmHg compared to 2.3 ± 0.6 mmHg for treated ($n = 6$) and control ($n = 3$) mice, respectively (Fig. 1, $P < 0.05$, t test). Control results correlated well with previous observations of deep hypoxia in this tumor model (17), precluding any further reduction in pO_2 levels as possibly expected with L-thyroxine (mean tumor pO_2 of 2.8 ± 0.4 mmHg).

³ A. Hercbergs, High tumor response rate to radiation therapy in biochemically hypothyroid patients. Presented at the American Association for Cancer Research, 1997.

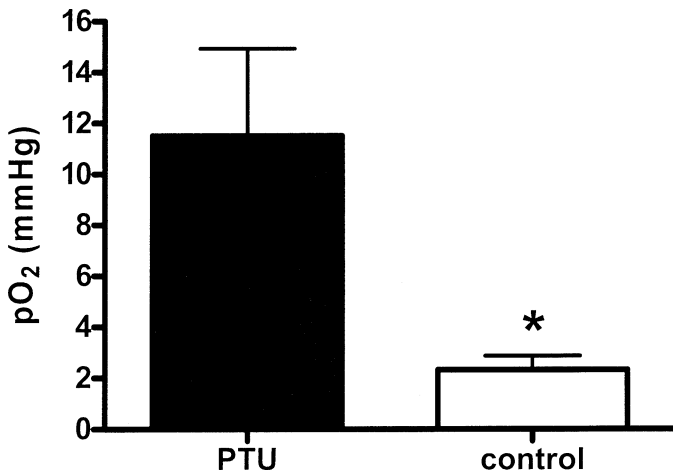


FIG. 1. Mean TLT tumor oxygenation in control ($n = 3$) and PTU-treated mice ($n = 6$) as measured by EPR oximetry.

Effect of Thyroid Status on Tumor Oxygen Consumption

We measured the oxygen consumption rate of tumor cells extracted from hypothyroid, hyperthyroid and euthyroid mice. Cells from hypothyroid mice consumed oxygen more slowly than cells from control mice, which themselves consumed oxygen more slowly than cells extracted from hyperthyroid mice. The mean slopes were $-0.86 \pm 0.16 \mu\text{M}/\text{min}$ ($n = 6$), $-1.41 \pm 0.11 \mu\text{M}/\text{min}$ ($n = 11$), and $-1.91 \mu\text{M}/\text{min} \pm 0.15 \mu\text{M}/\text{min}$ ($n = 9$), respectively (Fig. 2), and were all significantly different from each other (one-way ANOVA, Tukey's multiple comparison post-hoc test, $P < 0.001$).

Effect of Thyroid Status on Tumor Radiation Sensitivity

To determine whether thyroid status had an effect on tumor response to radiotherapy, hypo-, hyper- and euthyroid tumor-bearing mice were irradiated with 10 Gy of X rays and the tumor regrowth delays were measured. The corresponding control groups (nonirradiated mice) were also included in the study and showed no effect of PTU and L-thyroxine on the growth of TLT tumors between 8 and 16 mm in diameter. It should be noted, however, that the time to reach 8 mm in tumor diameter in hypothyroid mice was consistently shorter than in hyperthyroid mice (6 days compared to 8 days, respectively).

After irradiation, the time to reach a 12-mm tumor diameter was 11.0 ± 1.2 days for euthyroid mice ($n = 8$), 11.0 ± 1.2 days for hyperthyroid mice ($P > 0.05$, $n = 8$), and 17.2 ± 2.5 days for hypothyroid mice ($P < 0.05$, $n = 8$, one-way ANOVA, Dunnett's multiple comparison test) (Fig. 3). Finally, to discriminate between an oxygen effect and a direct radiosensitizing effect, an additional *in vivo* regrowth delay experiment was repeated including three groups of five mice: one control group (I) that received 10 Gy of X rays alone, one group (II) of PTU-treated mice + 10 Gy of X rays, and one group (III) of PTU-treated mice whose legs had been temporarily ligated to induce complete

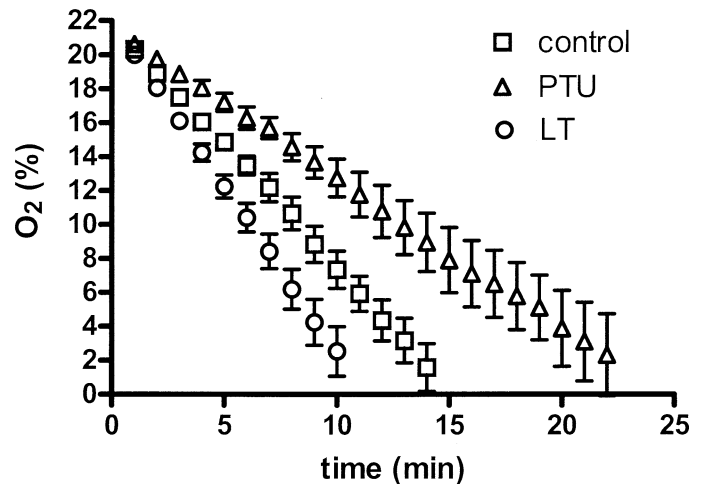


FIG. 2. Effect of PTU and L-thyroxine on TLT tumor cell oxygen consumption rate.

hypoxia at the time of irradiation. This experiment showed that oxygen was necessary for PTU to induce an additional regrowth delay in comparison with X rays alone since regrowth delays were similar for groups I and III and significantly increased in group II (Fig. 4).

DISCUSSION

The major findings of this study are the following: (1) Thyroid status significantly influences the response to radiotherapy; (2) the mechanism for this modulation in radiosensitivity involves a change in tumor oxygenation that is induced by a change in oxygen consumption by tumor cells.

Our results clearly indicate that thyroid hormones play a major role in the metabolism of tumor cells by modifying

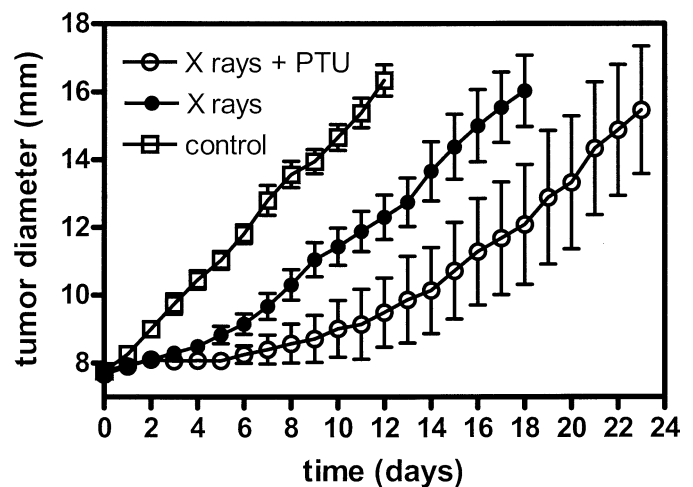


FIG. 3. Effect of hypothyroidism (PTU-treated mice) on TLT tumor regrowth. Mice were untreated (control), treated with 10 Gy of X rays, or treated with PTU in drinking water for 3 weeks before irradiation with 10 Gy of X rays. Each point represents the mean tumor diameter \pm SEM ($n = 8/\text{group}$).

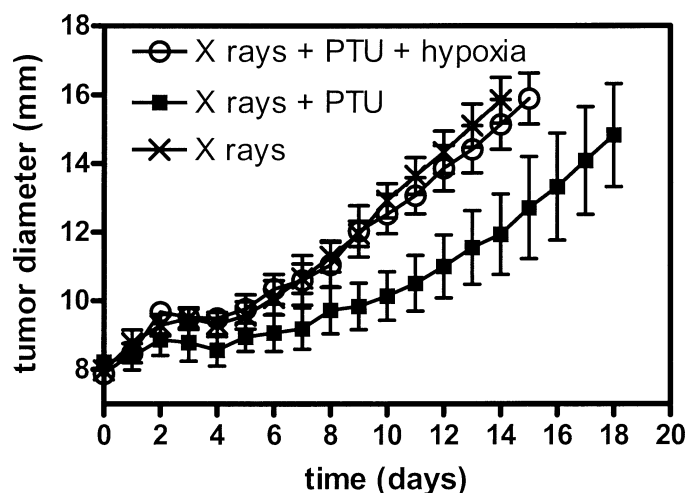


FIG. 4. Effect of tumor clamping at the time of irradiation on TLT tumor regrowth. Mice were irradiated with 10 Gy of X rays, with PTU in drinking water for 3 weeks before irradiation with 10 Gy of X-rays, or with PTU in drinking water for 3 weeks before irradiation with 10 Gy of X rays and with tumor ligation at the time of irradiation.

the rate of oxygen consumption. This mechanism is particularly relevant to explain differences in response to radiation and seems an important additional action of thyroid hormones besides their effect on tumor growth. In the model we used for the present study, we did not observe any direct effect of thyroid status on tumor growth except a slight delay in the time for tumors to reach 8 mm in diameter. This is probably because of the fast-growing properties of these experimental tumors. However, we could demonstrate a dramatic effect of thyroid status on tumor response to radiation therapy, with a regrowth delay factor of 1.6 in hypothyroid compared with euthyroid mice. The likely mechanism was a higher level of tumor oxygenation in hypothyroid mice, as shown with *in vivo* EPR oximetry. The involvement of oxygen in the radiosensitization process is demonstrated by the abolishment of such an effect when irradiation is carried out in temporarily ligated tumors. This is the result of a different tumor metabolism in hypothyroid mice: Oxygen consumption has been shown to be significantly decreased in PTU-treated mice. However, hyperthyroid mice show an increase in tumor oxygen consumption rate, which could result in a lower oxygenation level and thereby a decrease in tumor radiation sensitivity. We were not able to show this effect in a tumor model that is already highly hypoxic. We can, however, speculate that tumors from hyperthyroid mice would be more resistant to radiation in a less hypoxic tumor model since an increase in tumor oxygen consumption rate was observed. This could have significant implications in the clinical situation where human tumors are less hypoxic than experimental tumors (22).

It would be interesting to further characterize the mechanism(s) by which thyroid hormones are able to interact with the tumor cell line on a cellular and molecular level. At this stage, we do not know if the plasma membrane

receptor on integrin $\alpha_v\beta_3$ described to be involved in the action of thyroid hormones on the growth of glioma cells is also responsible for the radiosensitizing properties of the hormones (12).

In 1996, Hercbergs *et al.* suggested that induction of a clinically tolerable hypothyroid state in patients could become an integral part of the medical care of advanced cancers in conjunction with chemotherapy (1). These authors suggested that thyroid hormones can act on tumor metabolism, and particularly on mitochondrial biogenesis and membrane potential.³ Here we further demonstrate that a transient induction in hypothyroidism during the course of a radiotherapy regimen could be of significant benefit for cancer patients. Importantly, we also suggest that correction of a hyperthyroid state would be of crucial importance before starting radiation therapy. Finally, we provide a rationale for these observations by showing the involvement of tumor metabolic parameters, such as oxygen consumption rate.

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