In Vivo Oxygen Consumption Rate of DS Sarcoma Cells on Inhibition of DNA Synthesis

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Abstract

The effect of inhibiting DNA synthesis on the cellular O2 consumption rate of tumor cells (DS sarcoma) in vivo was analyzed using a photometric technique. Five days after DS-sarcoma ascites was induced in SD rats, animals were treated either with fludarabine (400 mg/kg i.p., 6 h prior to measurements) or lovastatin (3 × 20 mg/kg i.p., 24, 15, and 3 h prior to measurements), drugs that can inhibit tumor cell proliferation. In addition to cellular O2 consumption, the cell cycle distribution and the fraction of DNA-synthesizing cells in the tumor ascites were measured. Both drugs lowered DNA synthesis significantly, an effect that was more pronounced with fludarabine. The cellular O2 consumption rate following lovastatin application was significantly impaired (approximately 33%), whereas fludarabine had practically no effect on the respiration rate of tumor cells.

Introduction

Because tumor hypoxia is an important factor influencing the therapeutic outcome of standard radiotherapy, O2-dependent chemotherapy (1), and the expression of aggressive phenotypes (2), many attempts have been made to improve tumor tissue oxygenation. Because tumor oxygenation is the result of a dynamic steady state between O2 supply and O2 consumption of the tumor tissue, hypoxia could be reduced either by increasing the O2 supply or by reducing the O2 demand of the tumor cells. Most attempts to improve tumor oxygenation have tried to increase the O2 supply by: (a) increasing the arterial O2 pressure (e.g., by breathing normobaric or hyperbaric hyperoxic gases: Refs. 3 and 4); (b) improving (and homogenizing) the tumor perfusion (e.g., by increasing the perfusion pressure or by enhancing the flow properties of the blood; Refs. 3 and 5); or (c) enhancing the O2 release from blood into the tissue by shifting the O2-hemoglobin dissociation curve to the right (e.g., Ref. 6).

Theoretical studies (7) showed that a relatively small decrease in the O2 consumption rate of approximately 30% should be sufficient to drastically reduce hypoxia in the tumor and that this way of improving tumor oxygenation should be much more effective than increasing the arterial O2 supply or other measures. Because most of the energy and O2 consumed is used for cell division (especially in fast-growing tumors), an inhibition of DNA synthesis should lead to a significant reduction in cellular O2 consumption and, in turn, should improve tumor oxygenation, resulting in increased radiosensitivity. Therefore, two drugs, lovastatin and fludarabine, which can inhibit proliferation of tumor cells (8–11) and DNA synthesis (8, 9, 12), have been studied for their effect on cellular O2 consumption in an experimental sarcoma cell line of the rat in vivo. Fludarabine inhibits many enzymes required for DNA synthesis, e.g., ribonucleotide reductase, DNA polymerase α, δ, or ε, DNA helicase, and DNA ligase I (13), whereas lovastatin can inhibit DNA synthesis by blocking HMG-CoA3 reductase, resulting in a reduced mevalonate synthesis, leading to an impaired farnesylation of p21ras, which normally acts as a signal transducer for controlling the cell cycle (14–17). By this process, lovastatin leads to impaired cell cycle control, resulting in a partial G1 block (8). In addition to this, lovastatin reduces the cholesterol biosynthesis required for cell membrane formation. Because both drugs have comparable effects on DNA synthesis, the effects of these substances on cellular respiration rates were investigated.

Materials and Methods

Animals and Tumors. A hemorrhagic tumor ascites was induced in male Sprague-Dawley rats (Charles River Wiga, Sulzfeld, Germany; body wt, 170–320 g) by i.p. injection of 1 ml DS sarcoma cell suspension (approximately 106 cells/ml). Animals were allowed access to food and acidified water ad libitum throughout the investigation. After 5–6 days, when the animals had developed adequate ascites, they were randomly assigned to one of four experimental groups: two groups (verum and control) for lovastatin and fludarabine, respectively. All experimentation had previously been approved by the regional animal ethics committee.

Treatments. Twenty mg lovastatin (MSD, Sharp & Dohme, Haar, Germany) were dissolved in 1 ml DMSO (Sigma-Aldrich Chemie, Deisenhofen, Germany) and 2 ml PEG (M, 400; PEG 400; Sigma-Aldrich); the resulting solution had a concentration of 6.67 mg lovastatin/ml. Animals were treated with three i.p. injections of lovastatin (20 mg/kg body wt) 24, 15, and 3 h prior to the measurements. Animals treated only with the solvent (1 ml DMSO and 2 ml PEG 400/kg body wt) served as controls.

Fludarabine (Schering, Berlin, Germany) was reconstituted in calcium- and magnesium-free PBS, adjusted to a pH of 6.8–7.2, at a concentration of 40 mg/ml. The solution was filtered through a 0.2-µm Millipore filter. Animals were treated with single i.p. injections (400 mg/kg body wt) 6 h prior to measurements. Control animals were treated with equivalent volumes of PBS.

Measurement of O2 Consumption Rates. The O2 consumption rate of an ascites sample was analyzed using a photometric technique (18). The ascites (2 ml) was equilibrated in a thermostated tonometer with water-saturated carbon (95% O2 and 5% CO2) for at least 15 min. Thereafter, 50 µl of this sample were aspirated in a gas-tight syringe (Hamilton, Reno, Nevada). At precise time intervals of 5 min, 10-µl aliquots were injected from the syringe into an elution chamber, where cells were lysed, and all O2 contained in the injected volume was washed out by an O2-free nitrogen gas flow. The O2 concentration in this carrier gas was analyzed by an O2-sensitive dye in a continuously recording photometer (Zeiss, Oberkochen, Germany). By integrating the photometric reading, the O2 content of the injected aliquot was determined. Because the O2 content in the gas-tight syringe decreases due to the respiration by the cells, the consumption rate in the sample can be calculated from the time intervals between the aliquot injections and the decrease in the O2 content of the aliquots. The O2 consumption rate of the sample was normalized to the cell volume in the sample (by measuring the cytocrit using a standard microhematocrit technique) as well as to the number of cells in the sample, determined.

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The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; PEG, polyethylene glycol; wt, weight; BrdUrd, bromodeoxyuridine.

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by automatic cell counting in an EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, FL). Prior to and after each measurement series, the system was calibrated by injecting 5 µl room air containing a defined O₂ content (for more details on this technique, see Ref. 18).

**DNA Content and Proliferation Analysis.** After taking the first ascites sample for O₂ consumption measurement, the DNA-synthesizing cells were labeled with BrdUrd (100 mg/kg body wt; Boehringer Mannheim, Mannheim, Germany). BrdUrd was dissolved in PBS at a concentration of 10 mg/ml and injected i.p. After 60 min, a second ascites sample for DNA measurements was taken.

After lysing the erythrocytes in this sample, the tumor cells were washed twice with PBS containing 1 g glucose/liter. Afterward, the cells were fixed with ice-cold (–20°C) 70% ethanol and incubated for at least 30 min at 4°C. For determining the DNA content, the cells were centrifuged and resuspended in 1 ml PBS containing 1 g glucose/liter, 50 µg propidium iodide (Sigma-Aldrich Chemie), and 100 units of RNase A (Boehringer Mannheim). The cells were incubated for 10 min at 4°C and measured immediately afterward with a flow cytometer.

For the fluorescence antibody staining of BrdUrd incorporation, the DNA of the ethanol-fixed cells was denatured by resuspending the cells in 1 ml 2 M HCl containing 5% (v/v) Triton X-100 (Sigma-Aldrich) and incubating them at room temperature for 30 min. The cell pellet was then neutralized with Na-tetraborate (1 g liter H₂O; Sigma-Aldrich) and washed twice with PBS. For nucleus staining, the cells were resuspended in 100 µl PBS containing 0.5% (v/v) Tween 20 (Sigma-Aldrich) and incubated with 20 µl primary mouse anti-BrdUrd (Becton Dickinson, San Jose, CA) or polyclonal mouse IgG, (Becton Dickinson) as a negative control for 30 min at 4°C. After washing the cells once, the pellet was incubated with 5 µl secondary, goat antimouse, fluorescence-labeled antibody (Coulter) for 15 min at 4°C, followed by another washing step. Finally, the cells were incubated with propidium iodide, as described above, for 10 min at 4°C and measured immediately afterward. Flow cytometric measurements were performed using a EPICS Profile II flow cytometer, acquiring 10⁴ cells/sample. The fraction of DNA-synthesizing cells was determined from two-parameter scatter plots (relative DNA content versus log BrdUrd content) using a region of interest set in the isotypic negative control. The distribution of cell cycle phases was calculated from the measured histograms of 10⁴ cells using MultiCycle AV software (Phoenix Flow Systems, San Diego, CA).

**Statistical Analysis.** Results are expressed as means ± SEM. Differences between the groups were assessed by the two-tailed Wilcoxon test for unpaired samples. The significance level was set at α = 5% for all comparisons.

**Results**

The DS sarcoma ascites is a highly proliferating tumor. More than 40% of the cells are in the S-phase (Fig. 1), and 45–50% of the tumor cells are actively synthesizing DNA, as indicated by the fraction of BrdUrd-incorporating cells (Figs. 2 and 3). Fludarabine as well as lovastatin influence proliferation kinetics of DS sarcoma cells by significantly decreasing the fraction of cells in the S-phase. In addition, fludarabine increases the fraction of cells in G₂/M (Fig. 1). A more pronounced effect on the alteration of cell proliferation can be seen in the fraction of BrdUrd-incorporating cells. Fludarabine as well as lovastatin reduce the fraction of DNA-synthesizing cells significantly, although the extent of this reduction is different for the two substances. Fludarabine leads to an almost complete block of DNA synthesis (reduction of the fraction of DNA-synthesizing cells by more than 80%; Fig. 3), whereas lovastatin reduces this fraction only by about 40% of the control level (Fig. 2).

Although both substances have a strong impact on tumor cell DNA synthesis and proliferation kinetics, only lovastatin can reduce the O₂ consumption rate of the tumor cells. Lovastatin significantly reduces the cellular O₂ consumption by about 33% of the control level (Fig. 2), whereas fludarabine has no effect on the O₂ consumption rate (Fig. 3), although the arrest of DNA synthesis is much more effective with fludarabine than with lovastatin.

**Discussion**

The DS sarcoma ascites is a highly proliferating tumor, with almost 50% of the tumor cells actively synthesizing DNA. In accordance with previous in vitro and in vivo studies [8, 9, 12], both lovastatin and fludarabine can reduce DNA synthesis very effectively, although the inhibitory effect of fludarabine is more pronounced than that of lovastatin.

Fludarabine inhibits in vivo DNA synthesis almost completely and leads to a significant reduction of S-phase cells, as well as an increase of cells in G₂/M. These results are in good accordance with others (12).

Lovastatin also inhibits DNA synthesis by about 40% and significantly reduces the S-phase fraction. Additionally, the fraction of G₂/M cells was slightly, although not significantly, increased.

Jakóbisiak et al. (8) found in an in vitro study a cell cycle arrest in
early G₁ with a lovastatin concentration of 10 μM in the culture medium. It is difficult to predict what the effective mean lovastatin concentration in the present in vivo study was. Assuming that the pharmacokinetics of lovastatin is similar in humans and rats, the administered lovastatin dose (20 mg/kg body wt) would result in a plasma concentration of approximately 2 μM (9). At this lower concentration, only the inhibition of cell proliferation (8-10) and DNA synthesis (8, 9) were described in previous studies. In one of these studies, the G₁ fraction was found to be only slightly increased (8). Taking these considerations into account, the results of the present study are in accordance with others.

Although both substances have comparable inhibitory effects on DNA synthesis, only lovastatin reduced the cellular O₂ consumption rate by about 30%, whereas fludarabine has practically no effect on the respiration rate of tumor cells. From the fludarabine results, it can be concluded that a reduction in DNA synthesis does not necessarily result in a reduction in O₂ consumption in tumor cells.

Because only lovastatin reduces the cellular O₂ consumption, the conclusion can be drawn that other mechanisms can influence the respiration rate more effectively than inhibition of DNA synthesis. Lovastatin inhibits HMG-CoA-reductase, a microsomal enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis from HMG-CoA to mevalonic acid (14). Thus, lovastatin reduces cholesterol levels in proliferating cells, which is, e.g., required for cell membrane formation. Another product of mevalonic acid is farnesol, which binds to some intracellular proteins, e.g., ras proteins and other ras-related GTP-binding proteins (15-17). Farnesylated p21ras acts as a signal transducer for some growth factors (15-17). It seems probable that lovastatin alters several biochemical pathways (e.g., lipid and protein synthesis) by blocking the intracellular signaling through farnesylated p21ras. At present, however, it remains unclear by which mechanism lovastatin reduces the cellular O₂ consumption rate, although it seems that the inhibition of DNA synthesis is not responsible for this effect.

The O₂ consumption rate of a tumor also depends on the viability of the cells and on the extent of inherent necrosis within the tissue. With an increasing fraction of necrotic or apoptotic cells, which could be induced by different cytotoxic agents, the mean respiration rate of the tissue would decrease. However, in the ascites model used in this study, the fraction of dead cells is quite low. When viability was tested by trypan blue staining (data not shown), less than 1% of the cells were stained. Similar results were obtained when apoptosis was assessed from the fraction of cells with reduced DNA content (<2N). The fraction of dead or apoptotic cells never raised above 1% in any of the treatment or control groups (data not shown). Thus, the conclusion can be drawn that the reduction of the mean cellular O₂ consumption by lovastatin is not caused by the induction of necrosis or apoptosis in the tumor.

In addition to the use of lovastatin, several other possible mechanisms of reducing cellular O₂ consumption of tumors have been scrutinized over the last few years: (a) acute hyperglycemia (Crabtree effect; Ref. 19); (b) tissue hypothermia (20, 21); (c) application of calcium channel blockers (22, 23); and (d) narcotics, e.g., pentobarbital (24). However, all of these measures are unsuitable for a reduction of the respiration rate in solid tumors in vivo to improve the tumor oxygenation and, in turn, to increase the radiosensitivity of the tissue. The inhibition of cellular respiration by an acute hyperglycemia (Crabtree effect) is only a transient effect, which disappears almost completely 10 min after the start of a glucose infusion (19). Tissue hypothermia decreases the cellular O₂ consumption effectively (20). However, the improvement in tumor oxygenation is smaller than expected from the reduction of the respiration rate, due to: (a) constriction of vessels feeding the tumor; (b) an increase in blood viscosity, which, together with constriction of vessels, results in a reduced arterial blood supply to the tumor (21); and (c) a shift of the O₂-hemoglobin dissociation curve to the left, resulting in hampered O₂ release from erythrocytes to the tissue. These three mechanisms reduce the O₂ supply to the tumor cell and thereby diminish the effect of hypothermia. Calcium channel blockers (22, 23) and pentobarbital (24) show only minor effects on the O₂ consumption rate at concentrations tolerable in vivo. Only at higher, unphysiological concentrations in vitro is the impairment of the respiration rate more pronounced (22-24).

A significant decrease in cellular O₂ consumption of more than 30% can be achieved with lovastatin at concentrations that are tolerated in vivo. This substance, therefore, may bring about an improvement in the oxygenation status of hypoxic solid tumors and an increase in their sensitivity toward standard radiotherapy or O₂-dependent chemotherapy. Because lovastatin significantly reduces the fraction of S-phase cells, an improvement of cell cycle-dependent radiosensitivity may be expected (1), in addition to that obtained through the O₂-sparing effect. From the results obtained with fludarabine treatment, it must be concluded that DNA synthesis is only of minor importance regarding the O₂ consumption of tumor cells in vivo. However, it should be stated that using lovastatin for reducing the cellular O₂ consumption and probably increasing the radiosensitivity of tumors is only a first step toward a novel tool to improve tumor oxygenation. At the moment, the question remains of whether lovastatin is suitable for use in radiotherapy. In the present study, only the effect of lovastatin on DNA synthesis and O₂ consumption has been analyzed; other possible biological effects, especially during longer treatment with this substance, have to be addressed in future experiments. Other pharmacological agents impairing O₂-consuming metabolic pathways (e.g., lipid or protein biosynthesis) could also lead to a pronounced reduction in O₂ consumption and may result in an improvement of oxygenation in solid tumors.

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