Enhancement of Oxidative Cell Injury and Antitumor Effects of Localized 44°C Hyperthermia upon Combination with Respiratory Hyperoxia and Xanthine Oxidase

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Abstract

The effects of respiratory hyperoxia (RH) and xanthine oxidase (XO) during localized hyperthermia (HT) were investigated by determining markers of oxidative damage to lipids and proteins and tumor growth. Anesthetized rats with s.c. DS-sarcomas underwent one of the following treatments: (a) localized saline-bath HT (60 min, 44°C); (b) HT + RH (100% O₂); and (c) HT + RH + XO (15 units/kg i.v.). Sham-treated animals served as controls. Tumors were investigated for: (a) thiobarbituric acid-reactive substance formation and protein-bound 4-hydroxynonenal, as indicators of lipid peroxidation; (b) reactive oxygen-mediated protein modifications; (c) apoptosis; and (d) tumor volume growth. Upon treatment, increases in thiobarbituric acid-reactive substances, protein-bound 4-hydroxynonenal, protein-associated carbonyl functions, and number of cells undergoing apoptosis were found in tumor tissue, together with an inhibition of tumor growth. When treatment groups were compared, effects in the group HT + RH + XO were generally more pronounced. These findings indicate that the antitumor effect of HT is at least partially mediated through the selective induction of lipid peroxidation and oxidative injury to tumor cells, leading to apoptosis. This effect was enhanced by adding RH or RH + XO, presumably due to enhanced tissue damage following an increased formation of reactive oxygen species, with higher levels of lipid peroxidation and protein oxidation.

Introduction

HT is presently undergoing intensive clinical investigation as a treatment modality for cancer in combination with other therapeutic measures (radiation and chemotherapy). However, the exact mechanisms responsible for the antitumor effect of exposure to elevated temperatures are still not yet fully understood and warrant further experimental investigation. The extent of cellular death after HT varies with the treatment temperature, increasing abruptly above 42°C (1, 2). At the cellular level, damage to nucleic acids, proteins, lipids, and breakdown of the cellular membrane or lysosomes may be responsible for cell death after HT. At the same time, HT has a profound effect on the functional and structural integrity of tumor microcirculation (3, 4). Both effects are believed to play a role in the effectiveness of HT in the treatment of tumors. In addition, it has been repeatedly shown that HT can induce oxidative stress in tissues because of increased production of ROS and/or promotion of cellular oxidation events (5–9). Such an increased formation of ROS during HT can represent a further major mechanism of cell injury and could in principle be exploited in hyperthermic treatments of cancer. ROS are in fact cytotoxic and have been shown to kill cells via apoptosis (10). The aim of this study was to investigate whether the antitumor effects of HT could be further enhanced by combining HT with two additional treatments (respiratory hyperoxia, with or without systemic application of XO) aimed at further increasing oxidative stress (using lipid peroxidation, protein oxidation, and apoptosis as indicators) to achieve greater toxicity in tumor cells.

Materials and Methods

Animals and Tumors. Male Sprague Dawley rats (Charles River Wiga, Sulzfeld, Germany; body weight, 200–300 g) housed in our animal care facility were used in this study. They received a standard diet and water ad libitum. Solid tumors grew s.c. after injection of DS-sarcoma ascites cells into the hind foot dorsum (0.4 ml; ~10⁴ cells/μl). Studies were previously approved by the regional Ethics Committee and were conducted according to the guidelines for good laboratory practice according to the German Law for Animal Protection of 1987.

Treatments. Six days after tumor implantation, when the tumor volume was 0.5 ± 0.02 ml, animals were anesthetized with sodium pentobarbital (40 mg/kg i.p., Nembutal; Sanofi Ceva, Paris, France) and were randomly allocated to treatment groups as follows: group I, HT; group II, HT + RH; group III, HT + RH + XO; and group IV, sham-treated control animals.

Local Tumor HT. A thermostatically controlled saline bath (0.9% NaCl) was covered with a polystyrene board as a platform on which rats lay in the ventral position during treatment. The tumor-bearing hind feet were immersed into the saline through holes in the polystyrene so that tumors were completely submerged for 60 min. The saline temperature used was 44.3°C, which resulted in a corresponding temperature of 44.0°C in the tumor center, as measured using a 250-μm needle-type thermocouple (type 2 ABAc025/100/TI/D; Philips, Kassel, Germany).

RH. Animals spontaneously breathed humidified pure oxygen via a loosely fitting face mask for the duration of HT treatment.

XO Administration. XO (Fluka, Neu-Ulm, Germany) was dissolved in saline and administered i.v. into a tail vein, 15 min before commencement of HT treatment at a dose of 15 units/kg (injection volume, 2 ml/kg body weight).

In a first series of experiments, tumors were excised with a scalpel blade immediately after treatment, placed in tissue embedding medium (GSV 1; Snee Technik, Mainz, Germany), rapidly cooled in liquid nitrogen, and stored at −80°C until further investigation, as described below. In a second series of experiments, animals were allowed to recover after treatment, and tumor volume was monitored for up to 16 days after treatment.

Biochemical Assessment of Lipid Peroxidation (TBARS Assay). The analysis of TBARS as a marker of lipid peroxidation was carried out as described previously (11). Tumor sections of 20 μm were cut in a cryostat.
lysed in 250 µl of solubilization buffer [10 mM Tris (pH 7.4), 0.9% NaCl, 0.1% SDS, and 0.25 unit/µl benzamidase] and centrifuged at 10,000 × g for 5 min at 4°C. For protein measurement, an aliquot of 40 µl was frozen at −20°C. Two hundred µl of tissue lysate (or standards) were mixed with 20 µl of 0.1% SDS, and 0.25 unit/fil benzonase and centrifuged at 10,000 × g for 5 min at 4°C. Tissue acid dissolved in 50 ml of 0.1 M NaOH) were added. The reaction mixture was then incubated at 90°C for 45 min. Formed TBARe was extracted once with 500 µl of orthophosphoric acid (0.2 M) and diluted with ultrapure water to suitable concentrations. Protein content was measured spectrophotometrically using the Bio-Rad DC Protein Assay, according to the manufacturer’s protocol (Bio-Tek FL500; Biotek) for microplate assay sample size.

Immunochemistry Detection of Protein-bound 4-HNE. The occurrence of lipid peroxidation in tumor tissue was confirmed histochemically by revealing the binding to protein of 4-HNE, a reactive and specific product of phospholipid peroxidation (12). Tumor sections (10 µm thick) were cut at −20°C in a cryostat and subsequently fixed in cold acetone for 20 min. After rehydration in PBS, sections were then incubated with the following reagents: (a) 1% FCS for blockade of nonspecific antibody-binding sites; (b) 1:100 anti-4-HNE-protein rabbit polyclonal antibodies (at 4°C), obtained as described; (c) 1:50 antibody-immunoglobulin antibody, biotinylated (Dako, Hamburg, Germany); and (d) 1:100 FITC-extravidin (Sigma, Deisenhofen, Germany). Glycerol (90% v/v) in Tris buffer (pH 8.8) was used as mounting medium.

Immunochemical Detection of Protein Oxidation (Detection of Protein-bound Carbonyl Functions as 2,4-DNPH Derivatives). Carbonyl group formation in proteins (protein oxidation) was detected specifically after derivatization with 2,4-DNPH by immunohistochemistry, as described previously (13), and analyzed by light microscopy. Tumor sections of 10 µm were cut at −20°C in a cryostat, fixed in an ethanol:diethylether mixture (1:1 v/v) for 15 min, and allowed to react overnight at room temperature with an ac 2,4-DNPH reagent [15 mM 2,4-DNPH dissolved in absolute ethanol containing 1.5% (v/v) concentrated sulfuric acid]. Thereafter, specimens were washed in absolute ethanol containing 1.5% (v/v) concentrated sulfuric acid and rehydrated. The 2,4-dinitrophenyl hydrazones formed in proteins were then detected by indirect peroxidase or alkaline phosphatase staining as follows. Specimens were incubated in a humid chamber on ice for 2 h with rabbit anti-dinitrophenyl polyclonal antiserum (Dako). To inhibit unspecific binding of secondary antibody, specimens were treated with normal sheep serum (Sigma) and subsequently incubated with peroxidase-labeled sheep anti-rabbit polyclonal antiserum (Boehringer-Mannheim, Mannheim, Germany) for 1 h at room temperature. Specific detection of secondary antibodies was performed by the addition of diaminobenzidine tetrachlorochloride, resulting in a brown precipitate at sites where protein-associated carbonyls were present. Additional sections were stained with an alkaline phosphatase-anti-alkaline phosphatase system (Dako), using new fuchsin as the chromogen substrate. Selected sections were counterstained with hematoxylin and examined by light microscopy (Axioplan; Zeiss, Oberkochen, Germany).

Histochemical Determination of Apoptosis (Terminal Deoxynucleotidyl Transferase-mediated Nick End Labeling Assay). Apoptosis in tumor tissue was detected using the "Apoptosis Detection System, Fluorescein" according to the manufacturer’s protocol (Promega Corp., Mannheim, Germany) for frozen tissue sections. Tumor sections of 10 µm were cut at −20°C in a cryostat and subsequently fixed with 4% freshly prepared merthiolate-free formaldehyde for 30 min at room temperature. Specimens were washed in PBS and permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate solution for 30 min at room temperature. Positive controls were prepared by incubating tissue sections with 0.2 unit/µl DNase I (Boehringer-Mannheim) in 40 mM Tris-HCl (pH 7.4), 10 mM NaCl, 6 mM MgCl2, and 10 mM CaCl2 for 10 min at room temperature. Specimens were equilibrated and incubated with the nucleotide mix and the terminal deoxynucleotidyl transferase enzyme according to the manufacturer’s protocol at 37°C for 1 h. Negative controls were treated identically but in the absence of the terminal deoxynucleotidyl transferase enzyme. Specimens were washed in two changes of sodium citrate-buffered saline and counterstained with DAPI. Samples were mounted inembedding medium (SlowFade Light; Molecular Probes, MoBiTec, Goettingen, Germany) and analyzed immediately under a fluorescence microscope (Axiophot; Zeiss) using a standard fluorescein and DAPI filter set.

Statistical Analysis. Values for malondialdehyde equivalents (TBARe) were found in tumor tissue from each experimental group were compared using the H-test according to Kruskal and Wallis. Further analysis of differences in TBARe and of differences in tumor volumes was assessed using the Wilcoxon test for unpaired samples. The significance level in all cases was set at α = 5%.

Results

Significant increases in the amounts of malondialdehyde equivalents (TBARe) were found in tumor tissue after the various treatments compared with control tumors (Fig. 1). The highest levels were found in tumors treated with HT + RH + XO; these values were significantly higher than those found with HT alone.

The immunohistochemical detection of oxidized proteins matched well with the extent of the liperoxidative process measured as malondialdehyde equivalents. Compared with the control group (Fig. 2A), an increase was observed in the amount of protein-bound carbonyls in tumors treated with HT (Fig. 2B). The other treatments resulted in further increases in the amount of protein carbonyls within the tumor sections, whereby the effect was greatest with HT + RH + XO (Fig. 2, C and D).

As shown in Fig. 2E, protein oxidation was found largely to involve plasma membrane proteins of tumor cells (inset). Areas of increased protein oxidation (Fig. 2E) corresponded, on adjacent sections, to sites of accumulation of protein-bound 4-HNE (Fig. 2F), thus documenting the occurrence of a bona fide lipid peroxidation process. On examination of further consecutive sections, aspects of cellular damage and death (acidophilia and nuclear condensation) were found to colocalize strictly with the described areas of protein oxidation and lipid peroxidation (Fig. 2G).

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![Fig. 1. Effect of HT alone (HT), HT + RH, and HT + RH + XO (15 units/kg i.v.) on lipid peroxidation (formation of TBARe) in DS-sarcoma tissue. Results are presented as means from eight tumors per treatment group; bars, SE. For each individual tumor, three independent samples were analyzed. **, P < 0.01; *, P < 0.05.](attachment://image.png)
Fig. 2. A–D, localization of oxidized proteins (brown stain) in cryosections of rat DS-sarcomas exposed to normothermia (sham-treated control animals; A), HT (B), HT + RH (C), and HT + RH + XO (15 units/kg i.v.; D). Accumulation of protein carbonyls is highest in tumor tissue exposed to HT + RH + XO administration, whereas in control animals, protein-associated carbonyls are detectable only in small foci. E–G, colocalization of protein oxidation (E and inset: indirect alkaline phosphatase staining of 2,4-DNPH-derivatized protein carbonyl functions) with lipid peroxidation (F: indirect immunofluorescence of protein-bound 4-hydroxynonenal) and cell damage (G) in tumor tissue exposed to HT + RH + XO treatment. Arrows, corresponding areas in the three consecutive cryosections shown. Bars, 50 μm.
Fig. 3. Detection of apoptotic cells (green) by fluorescence microscopy in rat DS-sarcoma tissue sections exposed to normothermia (control, E), HT (F), HT + RH (G), and HT + RH + XO (15 units/kg i.v.; H). Simultaneously, cryosections were counterstained with DAPI (blue) to detect all nucleoli in the tissue sections (A–D).
ENHANCEMENT OF OXIDATIVE CELL INJURY UPON HYPERTHERMIA

Discussion

It is well established that the exposure of tumor cells to HT above 42°C results in increased cell death (1, 2), and it has been hypothesized that hypothermic injury to cancer cells might be (at least partially) caused by the activation of oxidative stress processes, as documented in human liver cancer (5) and in nonneoplastic cells (6–9). In liver tissue exposed to hypothermic perfusion, several markers of oxidative injury have in fact been determined, including the oxidation and depletion of reduced glutathione (7) and the appearance of aldehydic products of lipid peroxidation (5, 8, 9). In some studies, the activation of tissue xanthine dehydrogenase to its oxidase form has been implicated as a major mechanism in the oxidative stress caused by HT (5–7). Indeed, HT has been shown to induce XO activation (5–7), and it is known that the interaction of XO with its substrate (hypoxanthine) is associated with the generation of superoxide anion as a by-product. XO has been described in tumor tissues (14). Hypoxanthine concentrations are also elevated in tumor tissues (15), because of impaired energy metabolism leading to accumulation of ATP as well as to degradation of nucleic acids after cell death, thus prompting the conditions for the production of significant amounts of superoxide during the XO reaction. The superoxide dismutase product, hydrogen peroxide, is a well-established apoptotic agent (10). On the other hand, the reaction of reactive oxygen species with low molecular weight iron complexes would easily result in the onset of lipid peroxidation (16), and indeed the release of such iron complexes is likely to occur during disaggregation of dead cells. Thus, a series of biochemical changes set into motion within tumor tissue by HT would concur to favor the occurrence of oxidative cell injury and apoptosis. The data reported here represent the first demonstration that: (a) HT can selectively induce lipid peroxidation in tumor tissue; (b) this effect is enhanced when HT is accompanied by RH, with a maximum effect when HT and RH are administered together with XO; and (c) increasing levels of oxidative stress and lipid peroxidation result in the induction of increasing degrees of apoptosis in tumor tissue, apparently leading to a decreased growth rate of the tumor mass. This interpretation is based on several observations:

(a) The levels of TBARS, biochemical indicators of peroxidative damage to lipids, were found to increase significantly and progressively in the treated tumors as compared with untreated ones, the highest values occurring in the HT + RH + XO group (Fig. 1), whereas no such modifications were appreciable in control muscle tissue from the same animals (data not shown).

(b) The occurrence of oxidative damage was confirmed by the detection of increasing levels of protein carbonyls in tumor tissue after the various treatments (Fig. 2). Carbonyl functions can in fact originate on protein as the result of a direct attack by ROS on amino acid side chains, a process often termed “protein oxidation” (17) and/or from the covalent binding of reactive aldehyde and carbonyl products of lipid peroxidation, such as malonaldehyde and 4-HNE (12). In the present study, increased levels of oxidative stress and lipid peroxidation result in the induction of increasing degrees of apoptosis in tumor tissue, apparently leading to a decreased growth rate of the tumor mass. This interpretation is based on several observations:

(c) Increased protein oxidation and lipid peroxidation were associated with the occurrence of cellular damage and apoptosis. As shown in Fig. 2G, acidophilia and nuclear condensation were detectable in cells in strict correspondence with areas where higher levels of protein carbonyls and protein-bound 4-HNE were present. The occurrence of apoptosis in cancer cells after HT has been described previously (18–20), and the
involvement of oxidative reactions in the apoptotic process has been described (10). In the present study, when the possible occurrence of apoptosis was investigated by histochemical means in transplanted DS-sarcomas, it was found that the various treatments, while inducing increasing levels of oxidative stress, also induced correspondingly increasing extents of apoptosis; the most marked effect being that after HT + RH + XO treatment (Fig. 3, G and H). Although the appearance of apoptosis as a function of time after HT was not examined in this study (and therefore the time of maximal apoptosis could not be determined), the terminal deoxynucleotidyl transferase-mediated nick end labeling assay demonstrated that DNA strand breaks were evident immediately after treatment. This is in accordance with the findings of Shchepotin et al. (19), who investigated the hyperthermic i.p. perfusion of verapamil and reported rapid appearance of apoptosis in colon cancer xenografts with maximum breakage at 2 h after treatment and with the findings of Moroi et al. (20), who found maximal apoptosis immediately after HT treatment, with maximal necrosis occurring at least 6 h after treatment. Other studies, however, have reported maximal apoptosis to occur at a later point in time. For example, Sakaguchi et al. (18) reported maximal apoptosis in the rat Ward colon carcinoma at 4 h after HT and in a rat fibrosarcoma at 8 h after HT. The time of maximal apoptosis after HT, therefore, appears to be variable and may differ depending on the tumor studied and the heat dose applied. In the present study, tumor growth decreased significantly with the treatments used, showing an inverse correlation with the different levels of oxidative stress induced, although the differences between the treatment groups in terms of tumor growth were smaller than might be expected when the changes in lipid peroxidation, protein oxidation, and apoptosis are considered. This apparent disparity is presently undergoing further investigation.

In conclusion, cell damage and apoptosis have been induced in tumor cells by HT through the stimulation of prooxidant reactions, and these effects were enhanced by the association of HT with respiratory hyperoxia and i.v. administration of XO. Based on the discussed alterations of purine metabolism in tumor tissues resulting in elevated levels of hypoxanthine, it can be envisaged that RH and administration of exogenous XO can enhance the effects of HT by maximizing the production of ROS within the tumor mass. Thus, the data presented underline the therapeutic potential of strategies aimed at a selective stimulation of oxidative cell injury and apoptosis in tumor tissue.

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References

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