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Original Contribution

BREATH ETHANE GENERATION DURING CLINICAL TOTAL BODY IRRADIATION AS A MARKER OF OXYGEN-FREE-RADICAL-MEDIATED LIPID PEROXIDATION: A CASE STUDY

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Abstract—Total body irradiation (TBI) is used therapeutically for treatment of leukemias and other malignancies of the hemopoietic system. Ionizing radiation produces oxygen free radicals that contribute to cytotoxicity. Breath collected from one patient undergoing therapeutic TBI showed measurable changes in levels of ethane during treatment. Breath ethane is a marker of lipid peroxidation of n-3 fatty acids. The TBI treatment involved 4 days of irradiation. The largest changes in breath ethane occurred on Day 2. The increased levels of breath ethane on Day 2 were correlated to clinical manifestations of toxicity. The correlation of the onset of gastrointestinal side effects with higher levels of breath ethane suggests that breath ethane may be a clinically useful measure of the toxicity of various TBI fractionation treatment protocols currently in use at different medical centers. The levels of breath ethane on the other days of treatment were lower, suggesting that the oxidative–antioxidative balance of the patient may be important in protection against free radical mediated injury. These results for a single patient suggest that breath ethane may be a promising approach to elucidate the role of antioxidants in clinical TBI and should be extended for verification to a larger volunteer patient population.

Keywords—Breath, Ethane, Free radical, Lipid peroxidation, Oxidative damage, Total body irradiation, Radiation toxicity

INTRODUCTION

Free radical formation is the key mechanism responsible for producing cytotoxicity when ionizing radiation is used therapeutically in the practice of clinical radiation oncology. It has been postulated that free radicals are responsible for two-thirds of the indirect damage to the cell.[1–3] Many biomolecules, including DNA, are targets of attack by these reactive species. The unsaturated fatty acids present in cell membranes are readily peroxidized when electrons generated by ionizing radiation are carried by oxygen-containing species. The degree of damage caused by oxygen free radicals to biomolecules is determined by the oxidative status of the cell and the levels of readily available intracellular free radical scavengers.

Herein we report a case study of a patient undergoing total body irradiation (TBI) prior to a bone marrow transplant. Breath samples were obtained before, during, and after TBI to quantify ethane in exhaled breath. Ionizing radiation is known to cause generation of free radicals in the irradiated tissue;[1–3] these radicals initiate lipid peroxidation.[4] Therefore, it would be expected that patients undergoing TBI would have higher than normal levels of lipid peroxidation. The oxidation of lipids containing n-3 fatty acids liberates ethane; lipids containing n-6 fatty acids liberate pentane.[4,5] Thus, the detection of ethane and/or pentane in exhaled breath is a promising technique for the noninvasive measurement of the instantaneous oxidative stress status (OSS) of a patient.[6–9] Our past experience[10–12] has shown that the quantification of ethane, rather than pentane, is a more useful indicator of OSS in patients with normal liver function, who metabolize pentane,[13] causing appreciable losses.

Herein we report the real-time measurements of breath ethane over a 4-day period for a patient who was undergoing TBI. We believe these levels of breath

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ethane provide the first measurements of the changing OSS of a patient during the radiation treatment. The ability to quantify free radical production rates and OSS may allow a systematic probe of the effect of differing treatment regimens on the therapeutic ratio of the radiation treatment.

Total body irradiation has a well-defined role in the clinical preparation for bone marrow transplantation. The principle of TBI is to kill tumor cells while eradicating hemopoietic stem cells and lymphoid cells that mediate donor marrow rejection. The strategy for TBI regimens is to deliver a treatment plan with good antineoplastic activity and acceptable toxicity. The biological effects of TBI are influenced by many factors, including total dose, fractionation, dose rate, and dose distribution.[14–16] An increased total dose of TBI results in greater antineoplastic activity as well as treatment-related toxicity. TBI given without bone marrow rescue is limited because the median lethal dose of radiation is about 300 cGy in humans. Once a modality only reserved for refractory patients, intensive cytoreductive chemotherapy followed by total body irradiation and bone marrow transplantation may be the treatment of choice for selected patients with early stage chronic myelogenous leukemia, acute leukemia, and high grade lymphomas.[14] In the early use of TBI, several institutions[17] used a single fraction of 1000 cGy. However, the single fraction technique is associated with increased incidence of radiation pneumonitis, and most medical centers now use a fractionated regimen, thereby reducing the incidence of pneumonitis. Moreover, reducing the dose rate (cGy/min) to levels approximately one-tenth of those for more typical radiation fields further serves to diminish the severity of the pulmonary and gastrointestinal complications.

Clinical experience with total body irradiation has demonstrated that the lung is the most important dose-limiting structure.[14] Interstitial pneumonitis will cause death in 50% of the patients who develop this injury.[17] Several strategies have been devised at major centers to decrease lung toxicity. The Johns Hopkins Oncology Center uses complete lung blocks on Day 3 of treatment. Memorial Sloan–Kettering Cancer Center uses a hyperfractionated (multiple fractions per day) TBI regimen with partial lung blocks. Both these approaches have reduced the incidence of pneumonitis as compared with single fractionation regimens.[17] A higher incidence of pneumonitis is seen in regimens that do not utilize lung shielding.[18–20]

If the lung is an important site of oxidative damage, modifying the protocol for total body irradiation administration may improve the therapeutic ratio and decrease lung toxicity. The goal of this study was to perform an exploratory study of a single patient to investigate the utility of breath ethane as a noninvasive, clinically relevant biomarker for monitoring free radical induced tissue injury. It is our expectation that the measurement of breath ethane may be useful for evaluating the efficacy of the use of radiation sensitizers and antioxidant therapy in the practice of radiation oncology.

METHODS

Patient characteristics

The study subject was a 44-year-old White man (101 kg) diagnosed (June 1993) with chronic myelogenous leukemia. The patient had a 4-month history of fatigue, lightheadedness, and dizziness. A routine complete blood count showed a white blood cell count of 71,000/μl. He had no other significant past medical history and was a nonsmoker. He was treated initially with oral hydroxyurea and allopurinol and awaited allogenic bone marrow transplantation. Pretransplant pulmonary function tests, blood gases, renal function, computerized tomography scans of the chest and abdomen were all within normal limits. At the time of treatment for TBI his hematocrit was 33. Informed consent was obtained prior to initiating the study. The study protocol was approved by the Joint Committee on Clinical Investigation of The Johns Hopkins Medical Institutions.

Total body irradiation

The therapeutic protocol was initiated by administration of cyclophosphamide (50 mg/kg IV) daily for 4 days. Total body irradiation began on Day 5 with 4 MV photons (Varian Clinac 4/100); 300 cGy delivered AP/PA daily at 9.6 cGy/min for 4 days to deliver a total dose of 1200 cGy. The treatment distance is set at 360 cm from the average midplane separation taken at the mediastinum, umbilicus, and the pelvis. The collimator is rotated 45° for a treatment portal length of 204 cm × 204 cm. To achieve this extended field length and to optimize beam divergence, the beam is directed toward a lateral wall and the patient is placed close to the wall in a semiflexed position. Because the dose is prescribed at an average midplane, there is dose inhomogeneity in the region of the head, neck, and lower extremities. Compensating materials manufactured from Lexan® plastic or rice bags are interposed between the patient and the radiation beam source to improve dose distribution. Full thickness lung blocks (3% transmission) are used to shield the lung on Day 3 only. Dose verification is accomplished with diodes on Day 1 of treatment. The daily treatment takes approximately 50 min and is delivered in six sessions. Midway through treatment, the patient's position is reversed to assure dose homogeneity and to treat the

opposite side. Breath samples were obtained before treatment and after each 6-min treatment interval; thus, seven breath collections were made on each of the 4 treatment days.

Breath collection

The reproducibility of the technique used to obtain the breath sample is fundamental to accurate breath analysis. Important aspects of sampling include the accurate measurement of the volume of respiratory gas and the repeatability of the exhaled breath collection method. Because exhaled breath of healthy subjects is chemically nonhomogeneous, sampling different parts of the expiratory phase reflects different respiratory functions. The composition of exhaled breath is approximately constant during the "alveolar plateau," after the dead-space air has been purged and until the expiratory phase is complete. In the laboratory setting, the profile concentration of carbon dioxide in exhaled breath can be used to signal the onset of the alveolar plateau. In the clinical setting we have determined that reproducible breath samples can be obtained by the use of an electronic metronome set at 20 beats per minute. The patient was instructed to inhale or exhale, as appropriate, with each beat (i.e., 10 breaths/min). The patient was allowed to acclimate his breathing pattern with the metronome for about 30 s, and then four complete breaths were collected starting 1 min after the beam had been switched off. Breath was collected during obligatory mouth breathing using a one-way nonrebreathing valve (Hans Rudolph, Inc., Kansas City, MO) connected by a short length (15 cm) of respiratory tubing to a gas tight three-way valve on a gas collection bag (22 L, five-layer bonded polymeric sampling bags, Calibrated Instruments, Inc., Ardsley, NY). The time for the collection of breath was recorded, and the volume of breath sampled was measured volumetrically. These two values were used to calculate the minute ventilation.

With previous patients we established that careful control of the rates and extents of inspiration and expiration were important because patients were inclined to hyperventilate during breath collection even when asked to breathe normally.[21] Breath samples were collected before treatment and after each field was treated. Room air was sampled before and after treatment to correct levels of breath ethane for ambient ethane; this correction was necessary because it was not possible to use hydrocarbon scrubbed free air in this clinical setting. Ambient air was not flushed from the lungs with purified air because the required washout time can range between 4 and 15 min[6] and would have unacceptably extended the treatment. For a similar reason, only four breaths were collected at each breath sampling point.

Breath analysis

A modification of the technique developed in our laboratory[10-12] was used to analyze exhaled breath. Briefly, 60-ml aliquots of collected exhaled breath or room air were concentrated in a stainless steel tube (15 cm, 1.65 mm o.d.; 1.19 mm i.d.) packed with 2,6-diphenyl-ρ-phenylene oxide (60-80 mesh Tenax TA®, Alltech Associates, Deerfield, IL). This collection tube was submerged in an ethanol/liquid nitrogen slush bath (−117°C) contained in a Dewar. This reduced temperature increased the distribution constant for the sorption of gases or volatile vapors, such as methane and ethane, on the adsorbent surface but did not trap nitrogen or oxygen. The collection tube was maintained in the liquid nitrogen slush bath for 6 min to allow the adsorbent to equilibrate to −117°C, and then 60 ml of collected gas was drawn over the adsorbent. The collection tube was connected to a six-port gas sampling valve (1.59 mm inlets, Valco Instruments Co. Inc., Houston, TX) in place of the standard loop. After the gas had been sampled, the liquid nitrogen slush bath was replaced with a heating block maintained at 135°C, and the concentrated gas sample was injected immediately onto the gas chromatographic column by rotation of the gas sampling valve. The collection tube and adsorbent reach 135°C in 30 s. Separation of the components of breath was performed by gas solid chromatography using a stainless steel column (1.83 m, 3.2 mm o.d., 2.16 mm id) packed with octadecyl bonded silica (Chemipack C18, 80-100 mesh, Alltech Associates, Deerfield, IL). The temperature program for this separation was: isothermal 35°C for 5 min, linear temperature gradient from 35°C to 215°C at 10°/min, and finally isothermal at 215°C for 13 min. Helium (20 ml/min) was used as the carrier gas, and flame ionization detection was employed. The analytical method was calibrated daily by injection of a standard mixture of straight chain alkanes (C1 through C6). We have demonstrated that a sample of 60 ml of exhaled breath has sufficient ethane for analysis at an accuracy of 0.1 ppb for ethane.[10-12] The concentration of breath ethane may be expressed as ppb (corrected for ambient levels of ethane) or as pmol/min-kg; the latter allows corrections for minute ventilation and body weight and permits interpatient comparisons to be made. To convert from ppb to pmol/min-kg, the following relationship is used: {pmol/min-kg = [ppb (10^{-9}L/L) × minute ventilation (L/min)]/[24.45 (L/mol) × (pmol/mol) 10^{-12} × body weight (kg)]}. The data shown in Table 1 are expressed in both units.

Table 1. Breath Collection Data for Complete Treatment

Sample	Minute Ventilation (L/min)	Breath Ethane	
		ppb	pmol/kg-min
Day 1			
Pretreatment	9,610	0.94	3.7
Post 1st treatment interval	15,580	0.31	1.9
Post 2nd treatment interval	10,080	11.1	45
Post 3rd treatment interval	13,080	2.3	12
Post 4th treatment interval	8,500	0.31	1.9
Post 5th treatment interval	9,270	9.4	35
Post 6th treatment interval	10,000	2.4	9.8
Day 2			
Pretreatment	13,800	1.1	6.2
Post 1st treatment interval	11,000	0.21	0.9
Post 2nd treatment interval	15,800	1.6	10
Post 3rd treatment interval	14,330	6.9	40
Post 4th treatment interval	9,050	8.5	31
Post 5th treatment interval	10,300	15.7	66
Post 6th treatment interval	12,800	16.3	84
Day 3			
Pretreatment	16,730	0.64	4.3
Post 1st treatment interval	16,890	4.9	34
Post 2nd treatment interval	12,580	4.5	23
Post 3rd treatment interval	15,560	2.5	16
Post 4th treatment interval	7,750	6.3	20
Post 5th treatment interval	8,310	4.0	14
Post 6th treatment interval	8,880	5.1	18
Day 4			
Pretreatment	14,830	0.1	0.6
Post 1st treatment interval	16,750	5.3	36
Post 2nd treatment interval	10,130	0.67	2.7
Post 3rd treatment interval	8,080	7.4	24
Post 4th treatment interval	9,830	7.7	31
Post 5th treatment interval	10,380	4.0	17
Post 6th treatment interval	9,250	4.8	18

RESULTS

The patient was alert, relaxed, and compliant with the breath collection protocol throughout the 4 days of treatment. He generally tolerated the TBI treatment well, experiencing gastrointestinal symptoms only on the evening of Day 2.

Table 1 shows the minute ventilation rates and the levels of exhaled breath ethane before treatment and after each treatment interval on Days 1 through 4. Even though the patient's rate of breathing was controlled by the use of the metronome, his breathing depth varied and produced changes in his minute ventilation. The duration of each radiation treatment interval is approximately 6 min, with approximately 2 min between the end of one treatment interval and the start of the next. Exhaled breath was collected after each treatment interval 1 min after the beam had been switched off. There was a longer delay (5 min) between Treatment Intervals 3 and 4 because of the time involved in repositioning the patient. Timelines that show the relationship between the treatment intervals and breath collection points for each day are shown schematically in Figure 1.

The daily pretreatment levels of breath ethane are shown in Figure 2. There is an initial rise in the pretreatment level of breath ethane on Day 2 and a subsequent steady decrease until the lowest value was reached on Day 4. The variation in breath ethane production of a normal human subject during sequential daily breath sampling is ± 10%.[21]

Several different profiles for ethane production are seen over the 4-day course of treatment. The exhaled ethane levels as a function of radiation treatment intervals for Day 1 of treatment show a biphasic profile. The ethane level increased after Treatment Interval 2 from the pretreatment level and decreased after Treatment Interval 3, and a similar rise and fall in breath ethane levels was observed after Treatment Intervals 4, 5, and 6. By Day 2, this profile is replaced by an almost linear increase in exhaled breath ethane levels during the Treatment Intervals 1 through 6 (Fig. 3).

On Day 3 when full lung blocking (3% transmission) was used on the anterior and posterior treatment intervals, the overall ethane production was decreased. During Day 3 there was a delay of approximately 20 min between Treatment Intervals 3 and 4 (Fig. 1) to allow the technicians to obtain radiographs to assure

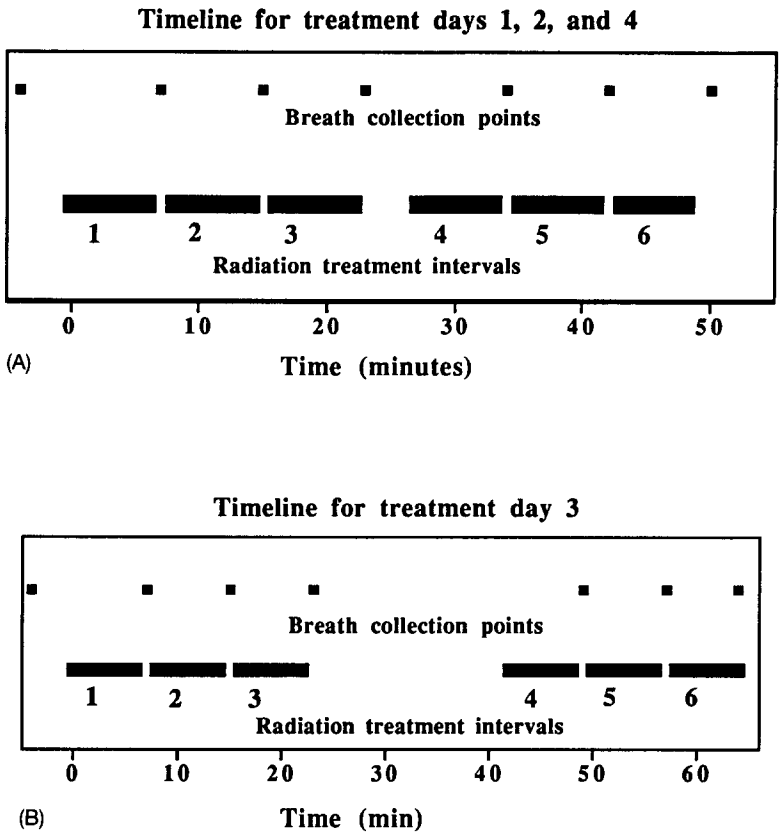

Fig. 1. Timelines showing the relationships between radiation treatment intervals, the breath collection points, and time on (A) Treatment Days 1, 2, and 4, and (B) for treatment Day 3.

ued proper positioning of the lungs blocks. The levels of ethane generated during the last day of treatment show reduced ethane production similar to that observed on Days 1 and 3.

The levels of breath ethane as a function of treatment interval and treatment day were examined statistically. Two-sided F-tests were used to compare the standard deviations of Day 2 to the standard deviations

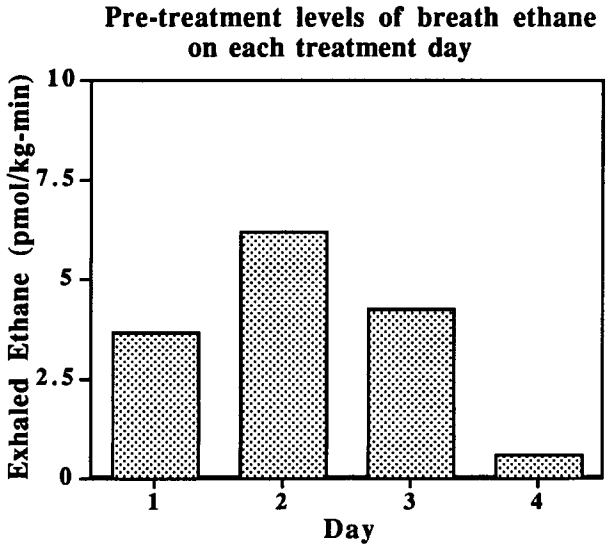

Fig. 2. The pretreatment levels of breath ethane on each of the four treatment days.

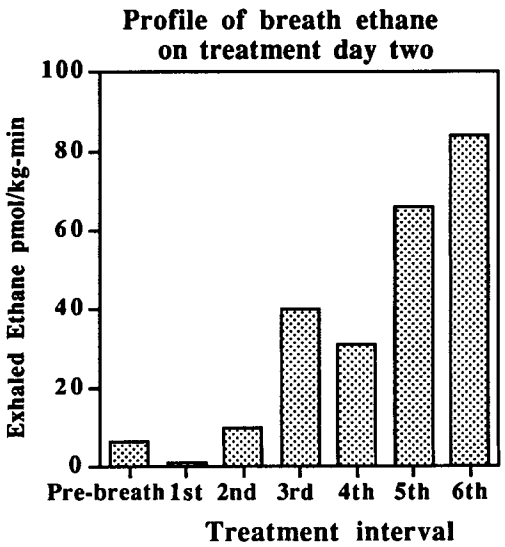

Fig. 3. The profile for breath ethane on Treatment Day 2 as a function of treatment interval.

for each of the other treatment days. The differences between the standard deviations for Day 2 and Day 1 were not statistically significant, whereas equivalent comparisons of the standard deviations for Day 2 and Day 3 and Day 2 and Day 4 were statistically significant ($p < 0.05$). The mean for Day 2 was compared to the means for treatment Days 1, 3, and 4 using t-tests. Only the differences between the means for Day 2 compared to Day 1 approached statistical significance.

Additional statistical analysis of the data was not possible because the exact breath collection protocol described herein had only been used on this single study subject. Previous studies using other patients undergoing radiation treatment had determined the time course of ethane production as a result of exposure to ionizing radiation. In these studies, breath was collected at different time points during treatment, after selected treatment intervals, and at differing times after radiation treatment.

DISCUSSION

The pretreatment levels of breath ethane on Days 1 through 4 show a notable increase at Day 2, and by Day 4 the pretreatment baseline ethane values have decreased to levels below that which is usually observed in healthy control patients (~3 pmol/kg-min).[21,22] These changes in daily breath ethane levels may reflect the oxidative stress status (OSS) of the patient. Therefore, daily breath ethane levels may be a noninvasive way to assess the physiological response to TBI.

There is a paucity of clinical information on the changes produced in OSS by total body irradiation. Clemens and coworkers[23,24] reported plasma levels, obtained before, during, and after therapy, of α- and γ-tocopherol, β-carotene and ascorbic acid in 22 patients receiving cytoreductive chemotherapy and bone marrow transplantation (18 patients received TBI). Blood plasma levels of lipid soluble antioxidants were observed to decrease after radiation by 20% for α- and γ-tocopherol and by 50% for β-carotene. This reduction was postulated to result from increased lipid peroxidation occurring during therapy. Because our TBI treatment regimen does not include supplementation with antioxidants, the reduction in the pretreatment level of breath ethane on Treatment Day 4 could be postulated to involve the upregulation of endogenous or extracellular antioxidant defenses to compensate for the reduction in circulating antioxidant vitamins occurring during radiation therapy.[25]

The profile of ethane generation on Day 1 shows an initial decrease below the baseline level after the first treatment interval and an increase in exhaled ethane after Treatment Interval 2; breath ethane level subsequently decreases after Treatment Interval 3. This profile of ethane generation with treatment interval is closely duplicated after Treatment Intervals 4, 5, and 6 when the patient is repositioned after Treatment Interval 3 (to treat the posterior field and improve the dose distribution). This biphasic profile was not observed on any other treatment day and cannot be explained until a larger patient population has been studied.

The level of breath ethane increases in an approximately dose-dependent manner on the second day of treatment (Fig. 3). It could be postulated that if the levels of antioxidant species and free radical scavengers are significantly depleted after the first treatment day, the treatment on the second day produces the maximum free radical damage. This patient presented with acute nausea and vomiting on the evening of Day 2; most patients undergoing TBI treatment with the treatment regimen described herein experience similar acute nausea and vomiting after the second day of treatment. Patients undergoing hyperfractionated treatments elsewhere experience similar symptoms after the first treatment day.[26] The correlation of the onset of gastrointestinal side effects with higher levels of breath ethane suggests that breath ethane may be a clinically useful measure of the toxicity of various TBI fractionation treatment protocols currently in use at different medical centers.

The activities of enzymes of the antioxidant system and lipid peroxidation products have been studied as a function of irradiation in the liver and thymus of rats.[27] Levels of glutathione reductase, glutathione transferase, and catalase were found to decrease significantly in the first 24 h after irradiation. Although this study did not report the effect of irradiation on subsequent days nor did it report levels of enzymes after the first 24 h, it supports our hypothesis that increased ethane production on Day 2 of the treatment may be due to reduced levels of antioxidant defense systems. Thus, dietary antioxidants might be most effective on Day 2.

Day 3 of therapy utilizes complete lung blocks during the entire treatment, and the overall ethane production rate is measurably lower than that which was observed on Day 2. This reduction in breath ethane levels, which potentially corresponds to lower overall lipid peroxidation rates, may be attributable to the use of lung blocks during treatment on Day 3. There are experimental data to suggest that lipid peroxidation contributes to the morphologic changes associated with pulmonary irradiation. Nozue and Ogatu[28] examined rat lungs after single fraction, single lung irradiation (300 cGy). Rats were sacrificed weekly until the end of the fifth week after irradiation. The untreated lung

appeared to have normal levels of lipid peroxides (as TBARS), but lipid peroxides drastically increased and marked degenerative cellular changes were found to have occurred in the irradiated lung in the third week after irradiation. Concentrations of enzymes in the antioxidant system (superoxide dismutase and glutathione) increased at the end of the fifth week after irradiation. These researchers showed a correlation between the morphologic changes and lipid peroxidative changes (as TBARS), suggesting that lung damage can be correlated with free radical injury.

The overall ethane production on Day 4 of therapy is comparable to that observed on Days 1 and 3 (when lung blocks were used), but was less than that observed on Day 2. This reduction in lipid peroxidation supports our suggestion that an upregulation of enzymes of the antioxidant system has occurred by Day 4. This hypothesis is supported by the marked reduction in pretreatment levels of breath ethane that is observed on Day 4 (Fig. 2) and by the observation that antioxidant enzymes increase postirradiation.[28] Superoxide, generated at reperfusion of an ischemic porcine liver, is the proximal mediator of heat shock gene (HSP-72) expression.[29] Thus, a total suppression of the early gene expression due to this reperfusion event could be achieved by the infusion of SOD. Breath ethane has been used to quantify free-radical-induced lipid peroxidation that occurs at reperfusion of the ischemic porcine liver,[11] and the infusion of SOD produced a similar significant attenuation of the levels of ethane produced. These two studies suggest that there is a relationship between oxidant injury, gene expression of HSP-72, and breath ethane levels. Reperfusion injury, a far more subtle cellular insult than that initiated by TBI, produced induction of heat-shock gene expression, which also involves induction of antioxidant defenses.[30] Therefore, we postulate that the observed decreases in breath ethane on Day 4 of TBI may involve upregulation of enzymes in the antioxidant system.

CONCLUSIONS

Breath ethane as a measure of lipid peroxidation caused by oxygen free radicals may be an important clinical tool to assess and quantify the degree of damage caused by ionizing radiation. Breath ethane allows for direct, real-time assessment of the oxidative stress status of these patients.[7–9] The usefulness of such noninvasive measurements may have an important role in the therapeutic use of antioxidant therapy, radiosensitizers, and radioprotectives in the management of radiation-induced toxicity. In this case study of total body irradiation we have observed that changes in breath ethane production occur over a course of treatment. We have also established an experimental protocol for breath collection whereby breath ethane can be used to follow TBI treatment in a clinical setting. If our observed changes in ethane production are typical of TBI treatment, they may be used in the future to guide the radiation treatment protocol to improve outcome or decrease toxicity.

Currently we are studying the oxidant stress status and its relationship to breath ethane levels as a result of TBI treatment in a larger patient population. The experimental protocol includes collection of daily pretreatment breath samples and simultaneous measurement of the oxygen-radical absorbance capacity.[31] If these studies show that the response is the same as that which we observed in this case study, we postulate that upregulation of antioxidant defenses could have important implications for the timing of administration of radiosensitizers, dietary antioxidants,[32] and radioprotective agents. We also plan to use breath ethane as a measure of OSS to study different radiotherapeutic regimens to improve the efficacy and reduce the toxicity of radiation treatment.

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ABBREVIATIONS

AP—anterior
cGy—centigray
PA—posterior
SOD—superoxide dismutase
TBI—total body irradiation