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Original Research Communication

Oxidative Stress and NF-κB Activation: Correlation in Patients Following Allogeneic Bone Marrow Transplantation

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ABSTRACT

Although *in vitro* data has linked reactive oxygen species (ROS) to activation of nuclear factor κB (NF-κB), little data exist regarding this relationship in human disease. We hypothesized that bone marrow transplantation (BMT) would impart a degree of oxidative stress that might lead to *in vivo* activation of the redox-sensitive transcription factor NF-κB. Because NF-κB regulates transcription of many proinflammatory mediators, we reasoned that activation of NF-κB might contribute to the development of transplant-related complications. To evaluate NF-κB activation in humans, we measured NF-κB binding activity in nuclear extracts of bronchoalveolar lavage (BAL) cells obtained before and after allogeneic bone marrow transplantation (BMT) in 7 patients. Changes in BAL cell NF-κB binding activity were compared with changes in urinary F_2-isoprostane concentration, an indicator of *in vivo* free radical-catalyzed lipid peroxidation. Although the extent of *in vivo* lipid peroxidation has substantial interindividual variability over time, we found a strong correlation between the pre/post-BMT ratio of urinary isoprostane concentrations and pre/post-BMT ratio of NF-κB binding activity in BAL cells, $R = 0.96$, $p = 0.0005$). This correlation is selective, because no relationship was found between the transcription factor CREB and urinary F_2-isoprostane excretion. Although limited by the small number of patients studied, our data link oxidant stress to NF-κB activation in human alveolar macrophages following BMT. It is possible that such interactions may contribute to the clinical course after BMT by affecting transcription of proinflammatory genes. Antiox. Redox Signal. 2, 93–102.

INTRODUCTION

NUCLEAR FACTOR κB (NF-κB) is a heterodimeric transcription factor that influences the expression of a variety of genes whose products include cytokines, growth factors, adhesion molecules, acute-phase proteins, and immunoreceptors (Siebenlist *et al.*, 1994). Transactivation of gene expression by NF-κB may be critical for immune function and generation of acute inflammation. A variety of agents have been shown to activate NF-κB in cells, including bacterial endotoxin, tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), viral proteins, ionizing radiation, and oxidative stress (Siebenlist *et al.*, 1994). Recently, generation of reactive oxygen species (ROS) has been linked to activation of NF-κB on the

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basis of four lines of evidence. First, treatment with hydrogen peroxide (H_2O_2) activates NF-κB in some cells (Schreck *et al.*, 1992; Los *et al.*, 1995). Second, agents that activate NF-κB in cells, including endotoxin, TNFα, IL-1β, and ionizing radiation, produce oxidative stress (Schreck and Bauerle, 1991; Adamson and Billings, 1992; Mohan and Meltz, 1994; Repine and Parsons, 1994). Third, antioxidants have been shown to inhibit NF-κB activation. Antioxidants suppress NF-κB activation in a variety of cell types in culture (Staal *et al.*, 1990; Schreck *et al.*, 1992; Ivanov *et al.*, 1993; Mohan and Meltz, 1994; Sen and Packer, 1996). *In vivo*, we have reported that the antioxidant *N*-acetylcysteine (NAC) inhibits NF-κB activation in lung tissue and attenuates endotoxin-induced neutrophilic lung inflammation in rats (Blackwell *et al.*, 1996). Finally, overexpression of endogenous antioxidant defenses has been shown to block NF-κB activation. Mirochnitchenko and Inouye (1996) have reported that peritoneal macrophages from transgenic mice overexpressing human Cu,Zn superoxide dismutase (SOD) have decreased NF-κB activation in response to phorbol 12-myristate 13-acetate. Also, Schmidt *et al.* (1995) reported that cell lines that overproduce catalase have diminished capability for NF-κB activation. In summary, these data indicate that oxidant stress may affect inflammation through activation of NF-κB.

An accurate estimate of the intensity of *in vivo* oxidant stress has been difficult to achieve (Halliwel and Grottveld, 1987). Isoprostanes are a family of compounds that are nonenzymatically generated during free-radical catalyzed lipid peroxidation of cellular phospholipids. Isoprostanes are structurally similar isomeric molecules that closely resemble prostaglandins. Low concentrations of F_2-isoprostanes and their metabolites can be detected in the urine and plasma of normal subjects. These levels are markedly elevated in animal models of oxidant stress, cigarette smokers, and patients meeting the criterion for the adult respiratory distress syndrome (ARDS) (Morrow and Roberts II, 1996; Carpenter *et al.*, 1997). When analyzed by gas chromatography with mass spectrometry (GC/MS) in urine, isoprostanes may be the most accurate indicator of whole-body cellular damage by oxidants. In this study, we have measured renal excretion of isoprostanes as a surrogate marker of *in vivo* oxidant stress.

Bone marrow transplantation, with attendant conditioning chemotherapy and radiation therapy, poses a severe stress on the endogenous antioxidant system. Several investigators have documented a fall in plasma levels of vitamin E and beta-carotene during induction therapy prior to bone marrow transplantation (BMT) (Clemens *et al.*, 1990). Durken and coworkers (1995) have shown a 40% decrease in "total radical-trapping antioxidant parameter of plasma," a functional assay of antioxidant defense. Also, certain chemotherapeutic agents, most notably carmustine (BCNU), directly inhibit the activity of glutathione peroxidase. In this setting, Hunnisett and colleagues found a rise in plasma lipoperoxides in patients undergoing BMT (Hunnisett *et al.*, 1995).

To define the relationship between activation of NF-κB and oxidant stress in humans, we measured NF-κB binding activity in nuclear extracts of bronchoalveolar lavage (BAL) cells in 7 patients before and after allogeneic BMT. We compared changes in NF-κB activation with changes in urinary F_2-isoprostanes. Our data show that oxidant stress is induced after inductive chemotherapy and BMT in most patients. We observed a close linkage between NF-κB binding activity in nuclear extracts from BAL cells and concurrent whole-body oxidant stress, as reflected by urinary concentrations of isoprostanes. Further research is necessary to relate the appearance of NF-κB gene products to the clinical course after BMT, including the development of idiopathic pneumonia syndrome (IPS).

MATERIALS AND METHODS

Patient selection

Seven patients were recruited from those undergoing allogeneic BMT at Vanderbilt University as part of the Bone Marrow Transplant-Lung Injury Following Engraftment (BMT-LIFE Study). The BMT-Life study is a prospective 4-year study aimed at understanding

mechanisms of a form of lung injury following BMT, known as IPS. Patients undergoing autologous transplants and peripheral stem cell transfers were not included in this study because they receive pharmacologic doses of the antioxidant, dimethyl sulfoxide (DMSO), used to cryopreserve bone marrow.

Bronchoscopy and bronchoalveolar lavage

Each patient underwent two bronchoscopies with BAL. The first BAL ($n = 7$) was performed in the operating room during placement of a Hickman catheter just before inductive chemotherapy (day −8). The second BAL was performed on either 1 day ($n = 3$) or 14 days ($n = 4$) following BMT (day +1 and +14, respectively). Human alveolar cells were obtained by fiberoptic bronchoscopy as outlined by the BAL study group (BAL Study Group, 1990). After informed consent and premedication with atropine, fentanyl, or midazolam, and with continuous monitoring of EKG and pulse oximetry, bronchoscopy was done with topical lidocaine anesthesia. Bronchoalveolar lavage of the right middle lobe or lingula occurred by instillation of 3–4 aliquots of 50 ml of sterile saline. Following each instillation, fluid was gently aspirated by manual suction with a 60-ml syringe.

Total and differential cell counts and cell preservation

BAL fluid was filtered through gauze and centrifuged at 500 × *g* for 10 min to separate cells from supernatant. Supernatant was saved separately and the cellular component was suspended in 10 ml of serum-free RPMI culture medium and placed on ice. Total cell counts were determined on a grid hemocytometer and differential cell counts were enumerated on cytocentrifuge slides stained with a modified Wright stain (Diff-Quick) by counting 400–600 cells in cross section. BAL cells were frozen after adding a sufficient amount of glycerol to the cellular suspension to result in a 20% final concentration. The samples were incubated on a rocker for 10 min at 4°C placed in a tight Styrofoam container, and then frozen at −70°C. Preliminary studies, using rat lavage cells, showed that this method was effective in preserving cells for extraction of nuclear and cytoplasmic proteins for at least 3 months (data not shown).

Preparation of nuclear protein extracts/ electrophoretic mobility shift assays

Nuclear protein extracts were done on BAL cells as described previously (Blackwell *et al.*, 1994). A consensus double-stranded NF-κB or CREB oligonucleotide (Stratagene, La Jolla, CA) were used as probes for elecrophoretic mobility shift assays (EMSA). End labeling was accomplished by treatment with T4 kinase in the presence of [^{32}P]ATP. Labeled oligonucleotide was purified on a Sephadex G-25 M column (Pharmacia Biotech, Inc., Piscataway, NJ). For NF-κB EMSA, 5 μg nuclear protein was added to a binding reaction mixture containing 10 m*M* Tris HCl, pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA, 0.2% Nonidet P-40, and 0.5 m*M* (DTT) (Stratagene) and a nonspecific blocker, salmon testis DNA (0.1 μg/μl) and incubated on ice for 15 min. For CREB EMSA, 5 μg of nuclear protein extract was added to a 16-μl volume of an identical incubation buffer (Stratagene) in a total volume of at least 25 μl. After incubation on ice, 5–10 × 10^5 cpm of labeled double-stranded oligonucleotide was added to each sample. This mixture was incubated at 25°C for 20 min and separated by electrophoresis on a 6% polyacrylamide gel (PAGE) in 1× TGE buffer. Gels were vacuum dried and subjected to autoradiography. Autoradiographs were later subjected to laser densitometry. Cold competition was done by adding 50 ng of specific unlabeled double-stranded probe to the reaction mixture. Nonspecific competition was done by adding 50 ng of unlabeled double-stranded mutant NF-κB oligonucleotide (5′-CCTGTGCTCCAATTTCCC TGGCCTGGA-3′) that does not bind NF-κB or CREB.

Isoprostane measurements

Spot urine samples were collected just before each bronchoscopy and frozen at −80°C before analysis. Urinary 8-iso-$PGF_{2\alpha}$ was quantified by modification of our previous published methods (Blackwell *et al.*, 1996). Briefly, 0.5 ng of [$^2H_4$]-8-iso-$PGF_{2\alpha}$ standard was added to 1 ml of urine and allowed to equilibrate. Fol-

lowing acidification to pH 3 with 1 *N* HCl, lipids were extracted with reverse-phase and straight-phase silica cartridges. After concentration, samples were purified by thin-layer chromatography (TLC) on LK6D plates with a mobile phase of chloroform/methanol/acetic acid/water (86:14:1:0.8, vol/vol/vol/vol). Samples were eluted from the appropriate silica segments, concentrated, and converted to the corresponding pentafluorobenzyl ester by addition of 40 μl of 10% pentafluorobenzyl bromide in acetonitrile and 20 μl of 10% *N,N*-diisopropylethylamine. Samples were then purified by TLC using the mobile phase of chloroform/ethanol (93:7, vol/vol). After concentration, samples were converted to the trimethylsilyl ether derivative by addition of 20 μl of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 20 μl of dimethyl formamide (DMF) and incubation at 40°C for 20 min. The sample was then dried and redissolved in undecane in preparation for GC/MS using a Varian-Vista instrument (Sunnyvale, CA) and a Nermag R1010C mass spectrometer (Fairfield, NJ). All assays for 8-iso-$PGF_{1\alpha}$ were performed by stable isotope dilution with GC/MS. In Fig. 1, a representative selected ion current profile (SICP) (from patient 1) used for quantification of 8-iso-$PGF_{1\alpha}$ is shown. The lower SICP at m/z 573 corresponds to the internal standard, [$^2H_4$]-8-iso-$PGF_{1\alpha}$, added to the initial sample to control for losses during derivatization. The upper SICP m/z 569 represents the isomeric F-ring compounds present in the sample. The location of 8-iso-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ are labeled. The quantity of 8-iso-$PGF_{1\alpha}$ was determined by

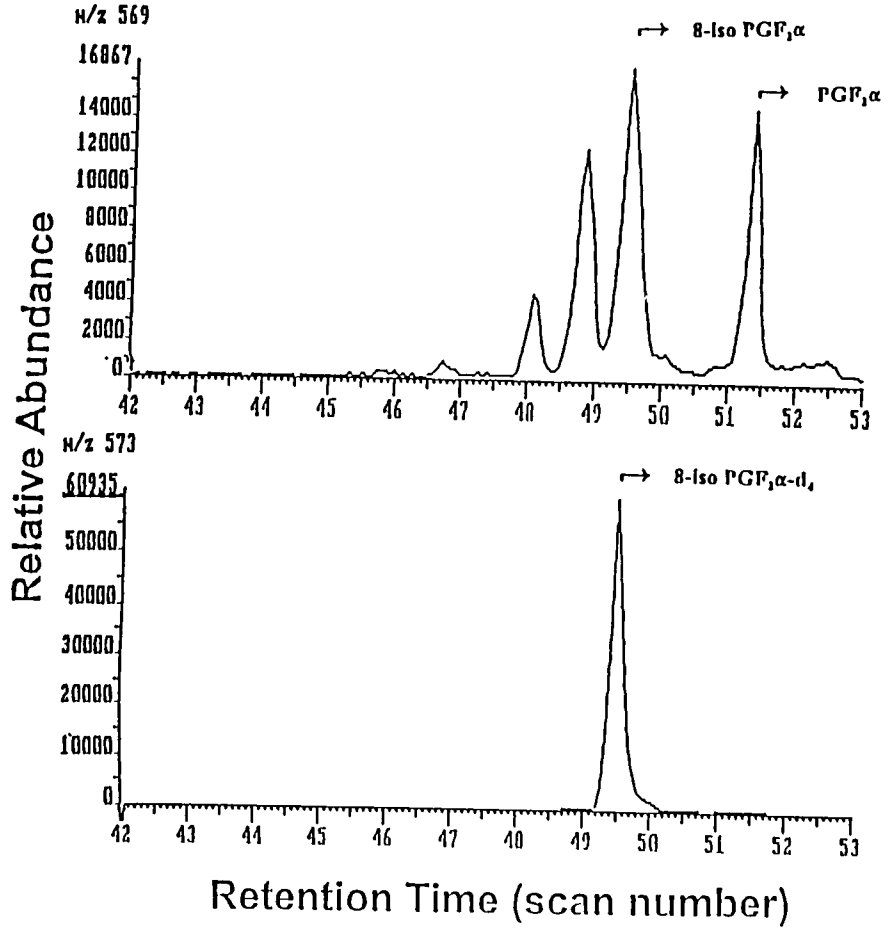

FIG. 1. Selected ion current profile (SICP) (from patient 1) used for quantification of 8-iso-$PGF_{1\alpha}$. The lower SICP at m/z 573 corresponds to the internal standard, 8-iso-$PGF_{1\alpha}$-d4 ([$^2H_4$]-8-iso-$PGF_{1\alpha}$), added to the initial sample to control for losses during derivatization. The upper SICP m/z 569 represents the isomeric F-ring compounds present in the sample. The location of 8-iso-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ are labeled. The quantity of 8-iso-$PGF_{1\alpha}$ was determined by comparison of the area under the elution curve to the internal standard.

comparing the peak area of the endogenous compound to that of the internal standard. Plasma isoprostanes, obtained at baseline at near the time of engraftment, were quantified using identical methods.

Statistical methods

Correlations between variables were sought using standard linear regression techniques (Fig. P Software, Durham, NC). The baseline and peak or periengraftment plasma isoprostanes were compared using Student's *t*-test.

RESULTS

Patient characteristics and BAL results

The mean age of the 4 men and 3 women enrolled was 43 ± 4 years. Five patients had chronic leukemia and 2 had non-Hodgkin's lymphoma as indications for BMT. Six patients received allogeneic transplants and 1 patient received a syngeneic graft. None of the patients received cells cryopreserved with DMSO. Four patients were active cigarette smokers before BMT and 3 patients were life-long nonsmokers. All 7 patients underwent the first bronchoscopy prior to beginning inductive chemotherapy. Three patients underwent the second bronchoscopy 1 day after marrow infusion and 4 patients underwent the second bronchoscopy 2 weeks after transplant. All 4 patients whose macrophages were collected at 14 days after transplant were in the immediate periengraftment period, with a mean peripheral blood leukocyte count of 0.4 ± 0.2 cells/mm^3. Three of these patients developed skin biopsy-proven graft-versus-host disease. The other patient developed both hepatic venoocclusive disease and idiopathic pneumonia syndrome with features of alveolar hemorrhage.

The total number of cells recovered by BAL was variable, but was not significantly different between the first and second bronchoscopies (48 ± 18 × 10^6 versus 33 ± 12 × 10^6 cells; p = ns). As expected, most of the BAL cells were alveolar macrophages at the time of the first BAL. The average counts (mean ± SD) were 96.5 ± 2.7% alveolar macrophages, 3.0 ± 2.7% lymphocytes, and 0.5 ± 0.6% neutrophils, and did not change significantly by the time of the second bronchoscopy (97.0 ± 4.3, 2.4 ± 3.7, and 2.1 ± 3.9, respectively).

Isoprostane measurements

Values of urinary 8-iso-PGF$_{1\alpha}$ obtained during the processes of induction chemotherapy, BMT, and engraftment (through day +14) are shown for 2 patients in Fig. 2. These 2 patients, both of whom had alveolar macrophages collected on day +14, are the patients who had the greatest change from baseline in excretion of isoprostanes in the sample obtained at the time of bronchoscopy. During the study, both patients increased excretion of isoprostanes by 4- to 5-fold; however, the degree of *in vivo* oxidant stress at the time of cell collection differed markedly between the 2 patients, especially when normalized to the baseline (361% and 24% of the baseline). Of the 7 patients studied, 6 had a rise above baseline in urinary 8-iso-PGF$_{1\alpha}$ concentrations at some point during chemotherapy, BMT, or engraftment. Additional analysis of mean plasma isoprostane values in 57 patients undergoing allogeneic BMT demonstrated a significant rise from 72 ± 1 pg/ml at study enrollment to 105 ± 1 pg/ml (mean ± SEM) in the periengraftment period (approximately day +14) ($p = 0.002$).

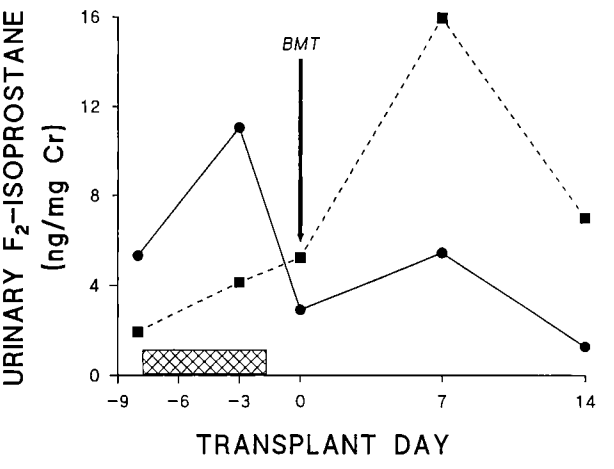

FIG. 2. Values of urinary 8-iso-PGF$_{1\alpha}$ [ng/mg Creatinine (Cr)] obtained during the processes of induction chemotherapy (a crosshatched bar), BMT (indicated by arrow), and engraftment (through day +14) are shown for two separate patients.

Measurement of NF-κB binding activity in nuclear protein extracts and correlation with urinary isoprostane concentration

Because *in vitro* studies have associated activation of NF-κB with the severity of oxidant stress, we sought evidence for a correlation between *in vivo* oxidant stress and NF-κB activation in humans. Figure 3 shows nuclear NF-κB binding activity in nuclear extracts of BAL cells obtained before and after BMT. The single specific NF-κB band detected in each sample is identified by an arrow on these two separate autoradiograms. The upper panel contains samples from the first and second BAL from patients 1–4 and the bottom panel contains samples from patients 5–7. The specificity of detected protein binding for the NF-κB motif

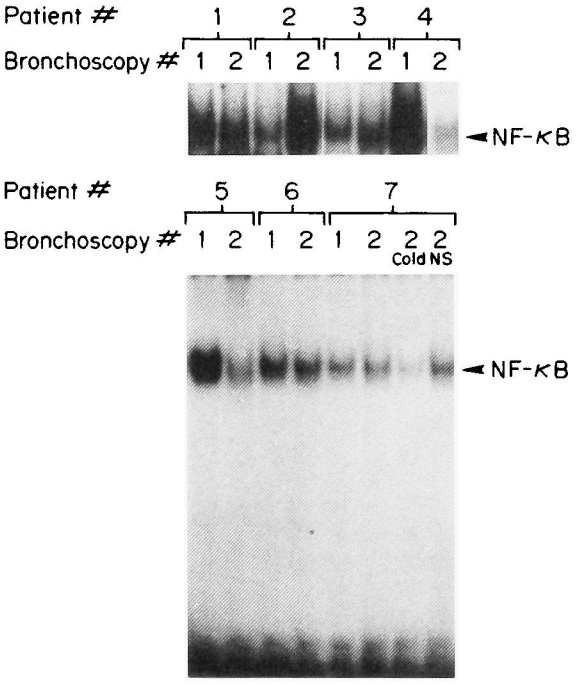

FIG. 3. Nuclear NF-κB binding activity in nuclear extracts of BAL cells obtained before and after BMT. The single specific NF-κB band detected in each sample is identified by an arrow on these two separate autoradiograms. The upper panel contains samples from the first and second BAL from patients 1–4 and the bottom panel contains samples from patient 5–7. Using samples from the second BAL from patient 7, the addition of 50 ng of unlabeled (cold) NF-κB probe completely blocked binding to the labeled NF-κB probe whereas the addition of 50 ng of a probe with a mutated NF-κB binding motif (NS, nonspecific) did not influence the banding pattern. Both BAL cell samples from each patient were processed at the same time and were run on the same EMSA.

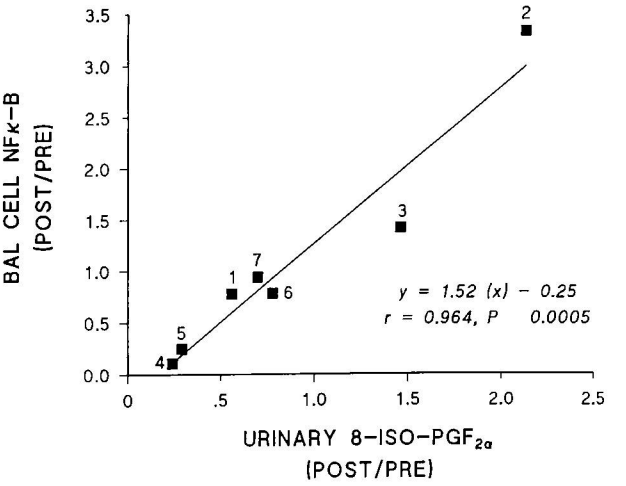

FIG. 4. Correlation of NF-κB binding activity and urinary concentrations of 8-iso-PGF$_{1\alpha}$. The intensity of each NF-κB band was measured using a laser densitometer and a ratio of NF-κB activation (post-BMT/pre-BMT) was calculated. Similarly, the ratio of 8-iso-PGF$_{1\alpha}$ (post-BMT/pre-BMT) was calculated from the spot urinary 8-iso-PGF$_{1\alpha}$ concentrations obtained just before the collection of each BAL sample. The relationship is significant ($r = 0.964, p = 0.0005$) between the urinary excretion of 8-iso-PGF$_{1\alpha}$ and BAL cell NF-κB binding activity.

was demonstrated by cold and nonspecific competition studies. Adding an excess of unlabeled (cold) NF-κB probe to nuclear protein extract from the second BAL cell sample from patient 7 completely blocked binding to the labeled NF-κB probe, whereas adding an identical amount of a probe with a mutated NF-κB binding motif (NS, nonspecific) did not influence the banding pattern. Both BAL cell samples from each patient were processed at the same time and were run on the same EMSA. The intensity of each NF-κB band was measured using a laser densitometer and a ratio of NF-κB activation (post-BMT/pre-BMT) was calculated. Similarly, the ratio of 8-iso-PGF$_{1\alpha}$ (post-BMT/pre-BMT) was calculated from the spot urinary 8-iso-PGF$_{1\alpha}$ concentrations obtained just before the collection of each BAL sample. Figure 4 shows the significant relationship ($r = 0.964$, $p = 0.0005$) between the urinary excretion of 8-iso-PGF$_{1\alpha}$ and BAL cell NF-κB binding activity.

We chose this method of presenting the data because the values for each patient can be normalized to their own baseline to correct for interindividual variability. In some patients, the NF-κB binding activity in nuclear extracts in-

creased while in other patients NF-κB binding activity decreased at the time of the second bronchoscopy. The significant relationship between NF-κB binding activity and 8-iso-$PGF_{1\alpha}$ excretion remains significant both for patients with reduction in systemic oxidant stress and in those with elevation at the time of the measurements.

Measurement of CREB and correlation with 8-iso-$PGF_{1\alpha}$

The relationship between NF-κB binding activity and 8-iso-$PGF_{1\alpha}$ excretion appears selective because there is no relationship between at least one other DNA-binding factor and 8-iso-$PGF_{1\alpha}$ excretion. Figure 5, A and B, shows representative EMSAs from patients 1, 5, 6, and 7 for binding activity of the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) in nuclear extracts of BAL cells samples. Figure 5a shows the CREB-binding pattern for nuclear extracts from the first and second BAL cell samples from patients 5–7 and Fig. 5b shows the CREB binding pattern from BAL cell samples from patient 1. Adding excess unlabeled probe to nuclear protein extract from the first BAL cell sample from patient 1 completely blocked binding to the labeled CREB probe. The addition of excess irrelevant probe containing a mutated NF-κB binding motif blocked only nonspecific binding to the labeled CREB probe. There was no relationship between the post-BMT/pre-BMT ratio for CREB binding in nuclear extracts of BAL cells and the post-BMT/pre-BMT ratio for urinary 8-iso-$PGF_{1\alpha}$ excretion (Fig. 6).

DISCUSSION

The major findings in our study include the observations that: (i) oxidant stress and lipid peroxidation are common events during the process of BMT, (ii) the severity of oxidant stress, as assessed by urinary isoprostane excretion, correlates closely with extent of activation of NF-κB in nuclear extracts of BAL cells,

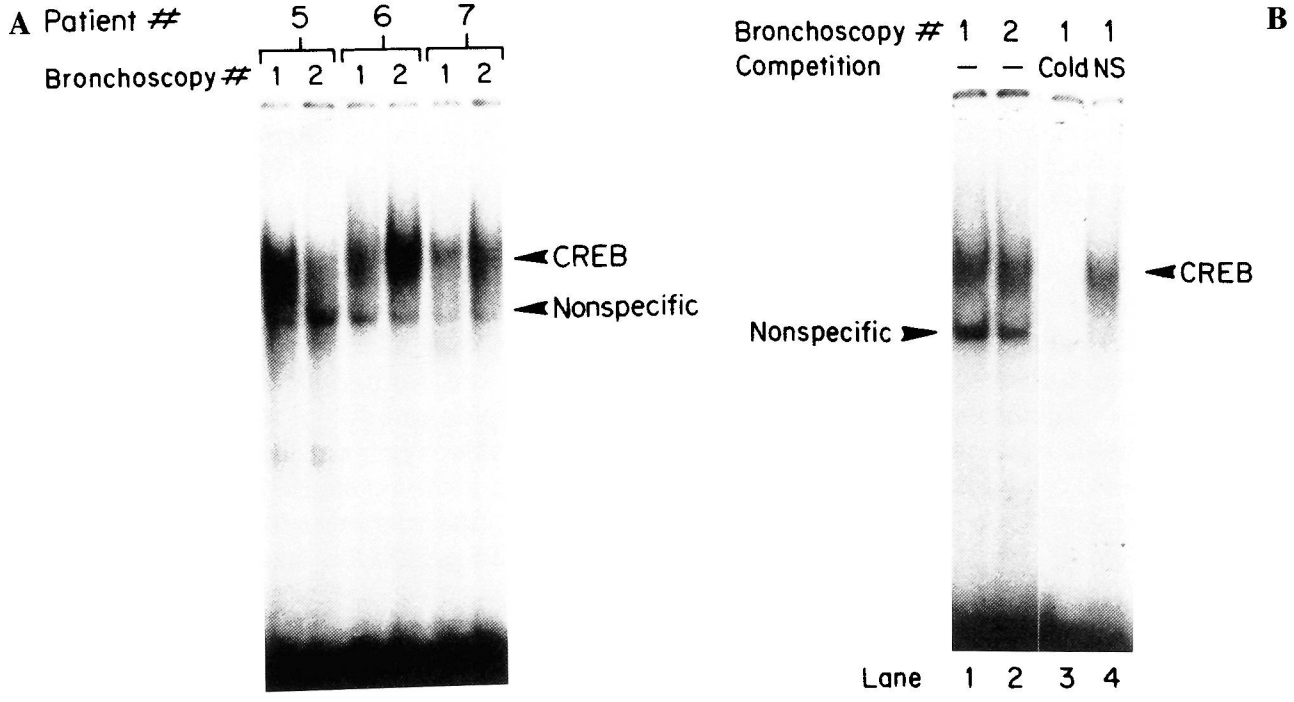

FIG. 5. EMSAs from patients 1, 5, 6, and 7 for binding activity of the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) in nuclear extracts of BAL cells samples. A. The CREB binding pattern for nuclear extracts from the first and second BAL cell samples from patients 5–7. **B.** CREB binding pattern from BAL cell samples from patient 1. Addition of 50 ng of an unlabeled probe completely blocked binding to the labeled CREB probe. The addition of 50 ng of an irrelevant probe containing a mutated NF-κB binding motif blocks only nonspecific binding to the labeled CREB probe.

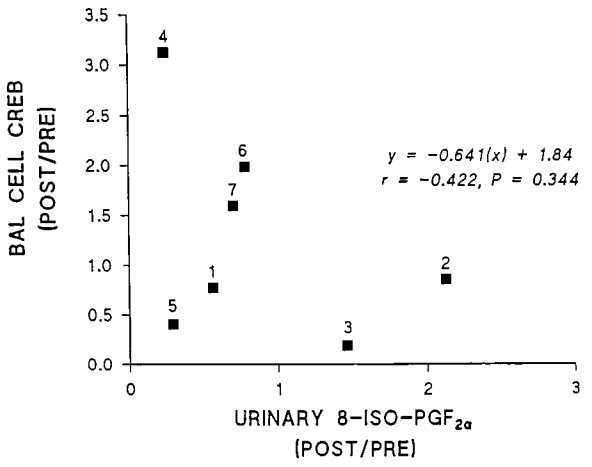

FIG. 6. No significant binding was found between the post-BMT/pre-BMT ratio for CREB binding in nuclear extracts of BAL cells and the post-BMT/pre-BMT ratio for urinary 8-iso-$PGF_{1\alpha}$ excretion. See Fig. 5 for definition of post-BMT/pre-BMT ratios.

and (iii) changes in BAL cell binding activity for CREB do not correlate with excretion of lipid peroxidation products or activation of NF-κB.

The role of NF-κB in regulating transcription of genes, such as TNFα, IL-8, cyclooxygenase-2, and 5-lipoxygenase, that modulate inflammation is well established in tissue culture experiments, but the significance of this factor for coordinating inflammation *in vivo* is unknown. Several recent studies have shown that NF-κB may play a vital role in lung inflammation (Blackwell *et al.*, 1994, 1996, 1997; Schwartz *et al.*, 1996; Haddad *et al.*, 1996; Shenkar *et al.*, 1996). Schwartz *et al.* (1996) have reported that NF-κB is activated in alveolar macrophages from patients with the ARDS to a higher degree than in alveolar macrophages from critically ill patients with other diseases. We have described a rat model of neutrophilic lung inflammation following intraperitoneal endotoxin injection in which endotoxin injection is followed by activation of NF-κB in alveolar macrophages and in lung tissue (Blackwell *et al.*, 1994, 1996). *In vivo* activation of NF-κB in this model correlates with chemokine gene expression. These events are followed by an influx of neutrophils into the alveolar space. Interventions such as pretreatment with antioxidants or induction of endotoxin tolerance block both NF-κB activation and NF-κB-dependent cytokine gene expression, and prevent neutrophilic lung inflammation (Blackwell *et al.*, 1996, 1997). Others have also shown the correlation between NF-κB activation and cytokine gene expression in lung tissue following an inflammatory stimulus. For example, Haddad *et al.* (1996) showed that ozone exposure induces NF-κB activation and chemokine gene expression in rat lungs that could be blocked by treatment with corticosteroids. In mouse lung tissue, NF-κB is activated by hypovolemic shock, which also leads to activation of cytokine production (Shenkar *et al.*, 1996). Taken together, these studies establish an *in vivo* link between NF-κB activation in the lung, cytokine production, and generation of lung inflammation. The activation state of NF-κB in BAL cells may reflect the concurrent capacity of these cells to produce a variety of inflammatory mediators, and could be a useful surrogate marker for *in vivo* cytokine production and inflammation in the lung and other organs.

We observed substantial variations of urinary excretion in F2-isoprostanes in patients undergoing chemotherapy, BMT, and engraftment. However, 6 of 7 patients had an increase in isoprostane excretion sometime during chemotherapy, BMT, or engraftment. At the time of the second bronchoscopy, some patients had a sustained increase in isoprostane excretion whereas in other patients it had returned to or below the baseline. Whether the levels of these compounds were increased or decreased at the time when alveolar macrophages were harvested, a close relationship with NF-κB activation remained. The isoprostanes are formed *in situ* on membrane phospholipids and do not require the presence of active cyclooxygenase, lipoxygenase, or p450 enzymes (Morrow *et al.*, 1992). Rather, their formation occurs via peroxidation of esterified arachidonic acid by a free radical-catalyzed process and results in the generation of four regioisomeric cyclic endoperoxide compounds resembling the unstable precursor prostaglandin G_2 (Morrow *et al.*, 1990). These compounds are subsequently reduced to generate isoprostanes. Although in these studies we have used the levels of these compounds as markers of *in vivo* oxidant stress, they are also potentially biologically active as spasmogens

for both vascular and airway smooth muscle (Kang *et al.*, 1993). Recent reports have described formation of not only D- and E-ring isoprostanes, but also isothromboxanes and isoleukotrienes (Morrow and Roberts II, 1996), which could be mediators of the clinical response to chemotherapy and BMT.

The current report was not designed, and is clearly underpowered, to examine the relationship between the biochemical parameters studied and either mortality or transplant-related complications. However, our information provides the first link in humans between the biochemical sequelae of lipid peroxidation and activation of NF-κB in the lung. Our data support the concept that cellular activation in the airspace is a multistep process in which alterations in the level of ROS affect cell membranes, downstream signaling events, gene transcription, and ultimately release of biologically active compounds that modulate inflammation and structural change. Such information may provide the theoretical foundation for the trial of antioxidants as prophylaxis against post-BMT complications.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ARDS, Acute respiratory distress syndrome; BAL, bronchoalveolar lavage; BMT, bone marrow transplantation; BSTFA, bis(trimethylsilyl)trifluoroacetamide; cAMP, cyclic adenosine monophosphate; Cr, creatinine; CREB, cyclic adenosine monophosphate response element binding; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; GC/MS, gas chromatography/mass spectrometry; H_2O_2, hydrogen peroxide; IL-1, interleukin-1; IPS, idiopathic pneumonia syndrome; NAC, *N*-acetylcysteine; NF-κB, Nuclear factor kappa B; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute; SICP, selected ion current profile; SOD, superoxide dismutase; TLC, thin-layer chromatography; TNF, tumor necrosis factor; 8-iso-PGF2a, 8-isoprostaglandin PGF2a.

REFERENCES

ADAMSON, G.M., and BILLINGS, R.E. (1992). Tumor necrosis factor induced oxidative stress in isolated mouse hepatocytes. Arch. Biochem. Biophys. **294,** 223–239.

BAL STUDY GROUP. (1990). Bronchoalveolar lavage constituents in healthy individuals, idiopathic pulmonary fibrosis, and selected comparison groups. Am. Rev. Respir. Dis. **141,** S169–S202.

BLACKWELL, T.S., HOLDEN, E.P., BLACKWELL, T.R., DELARCO, J.E., and CHRISTMAN, J.W. (1994). Cytokine-induced neutrophil chemoattractant mediates neutrophilic alveolitis in rats: association with nuclear factor kappa B. Am. J. Respir. Cell Mol. Biol. **11,** 464–472.

BLACKWELL, T.S., HOLDEN, E.P., BLACKWELL, T.R., CHRISTMAN, B.W., and CHRISTMAN, J.W. (1996). Activation of NF-κB in rat lungs by treatment with endotoxin: modulation by treatment with N-acetylcysteine. J. Immunol. **157,** 1630–1637.

BLACKWELL, T.S., BLACKWELL, T.R., and CHRISTMAN, J.W. (1997). Impaired activation of NF-κB in endotoxin-tolerant rats is associated with down-regulation of chemokine gene expression and inhibition of neutrophilic lung inflammation. J. Immunol. **158,** 5934–5940.

CARPENTER, C.T., PRICE, P., and CHRISTMAN, B.W. (1997). Exhaled breath condensate isoprostanes are elevated in patients with ALI/ARDS. Am. J. Respir. Crit. Care Med. **155,** A582.

CLEMENS, M.R., LADNER, C., EHNINGER, G., EINSELE, H., RENN, W., BUHLER, E., WALLER, H.D., and GAY, K.F. (1990). Plasma vitamin E and beta-carotene concentrations during radiochemotherapy preceding bone marrow transplantation. Am. J. Clin. Nutr. **51,** 216–219.

DURKEN, M., AGBENU, J., FINCKH, B., HUBNER, C., PICHLMEIER, U., ZELLER, W., WINKLER, K., ZANDER, A., and KOHLSCHUTTER, A. (1995). Deteriorating free radical-trapping capacity and antioxidant status in plasma during bone marrow transplantation. Bone Marrow Transpl. **15,** 757–762.

HADDAD, E.B., SALMON, M., KOTO, H., BARNES, P.J., ADCOCK, I., and CHUNG, K.F. (1996). Ozone induction of cytokine-induced neutrophil chemoattractant (CINC) and nuclear factor-kappa B in rat lung: inhibition by corticosteroids. FEBS Lett. **379,** 265–268.

HALLIWELL, B., and GROTTVELD, M. (1987). The measurement of free radical reactions in humans. FEBS Lett. **213,** 9–14.

HUNNISETT, A., DAVIES, S., McLAREN-HOWARD, J., GRAVETT, P., FINN, M., and GUERET-WARDE, D. (1995). Lipoperoxides as an index of free radical activity in bone marrow transplant recipients. Preliminary observations. Biol. Trace Element Res. **47,** 125–132.

IVANOV, V., MERKENSCHLAGER, M., and CEREDIG, R. (1993). Antioxidant treatment of thymic organ cultures decreases NF-κB and TCF1(α) transcription factor activities and inhibits $\alpha\beta$ T cell development. J. Immunol. **151,** 4694–4704.

KANG, H.K., MORROW, J.D., ROBERTS, L.J. II, NEWMAN, J.H., and BANNERJEE, M. (1993). Airway and vascular effects of 8-epi-$PGF_{2\alpha}$ in isolated perfused rat lung. J. Appl. Physiol. **74,** 460–465.

LOS, M., DROGE, W., STRICKER, K., BAEUERLE, P.A., and SCHULZE-OSTHOFF, K. (1995). Hydrogen peroxide as a potent activator of T lymphocyte functions. Eur. J. Immunol. **25,** 159–165.

MIROCHNITCHENKO, O., and INOUYE, M. (1996). Effect of overexpression of human Cu,Zn superoxide dismutase in transgenic mice on macrophage functions. J. Immunol. **156,** 1578–1586.

MOHAN, N., and MELTZ, M.D. (1994). Induction of nuclear factor κB after low-dose ionizing radiation involves a reactive oxygen intermediate signaling pathway. Radiat. Res. **140,** 97–104.

MORROW, J.D., and ROBERTS II, L.J. (1996). The isoprostanes: current knowledge and direction for future research. Biochem. Pharmacol. **51,** 1–9.

MORROW, J.D., HARRIS, T.H., and ROBERTS, L.J. II. (1990). Noncyclooxygenase oxidative formation of a series of novel prostaglandins: analytical ramifications for measurement of eicosanoids. Anal. Biochem. **184,** 1–10.

MORROW, J.D., AWAD, J.A., BOSS, H.J., BLAIR, I.A., and ROBERTS, L.J. II. (1992). Non-cyclooxygenase-derived prostanoids (F2-isoprostanes) are formed *in situ* on phospholipids. Proc. Natl. Acad. Sci. USA **89,** 10721–10725.

REPINE, J.E., and PARSONS, P.E. (1994). Oxidant-antioxidant balance in endotoxin-induced oxidative injury and tolerance to oxidative injury. In: *Endotoxin and the Lungs.* K.L. Brigham, ed. (Marcel Dekker, Inc., New York) pp. 207–227.

SCHMIDT, K.N., AMSTAD, P., CERUTTI, P., and BAEUERLE, P.A. (1995). The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-κB. Chem. Biol. **2,** 13–22.

SCHRECK, R., ALBERMAN, K., and BAEUERLE, P.A. (1992). Nuclear factor κB: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). Free Rad. Res. Commun. **17,** 221–237.

SCHRECK, R., and BAEUERLE, P.A. (1991). A role for oxygen radicals as second messengers. Trends Cell Biol. **1,** 39–42.

SCHRECK, R., GRASSMAN, R., FLECKENSTEIN, B., and BAEUERLE, P.A. (1992). Antioxidants selectively suppress activation of NF-κB by human T-cell leukemia virus type 1 Tax protein. J. Virol. **66,** 6288–6293.

SCHWARTZ, M.D., MOORE, E.E., MOORE, F.A., SHENKAR, R., MOINE, P., HAENEL, J.B., and ABRAHAM, E. (1996). Nuclear factor-κB is activated in alveolar macrophages from patients with acute respiratory distress syndrome. Crit. Care Med. **24,** 1285–1292.

SEN, C.K., and PACKER, L. (1996). Antioxidant and redox regulation of gene transcription. FASEB J. **10,** 709–720.

SHENKAR, R., SCHWARTZ, M.D., TERADA, L.S., REPINE, J.E., McCORD, J., and ABRAHAM, E. (1996). Hemorrhage activates NF-kappa-B in murine lung mononuclear cells *in vivo.* Am. J. Physiol.: Lung Cell. Molec. Physiol. **14,** L729–L735.

SIEBENLIST, U., FRANZOSO, G., and BROWN, K. (1994). Structure, regulation and function of NF-κB. Ann. Rev. Cell. Biol. **10,** 405–455.

STAAL, F.J.T., ROEDERER, M., HERZENBERG, L.A., and HERZENBERG, L.A. (1990). Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **87,** 9943–9947.

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2. J HADDAD. 2004. Hypoxia and the regulation of mitogen-activated protein kinases: gene transcription and the assessment of potential pharmacologic therapeutic interventions. *International Immunopharmacology* **4**:10-11, 1249-1285. [CrossRef]
3. John J Haddad. 2002. Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cellular Signalling* **14**:11, 879-897. [CrossRef]
4. Uwe Senftleben, Michael Karin. 2002. The IKK/NF-#B pathway. *Critical Care Medicine* **30**:Suppl., S18-S26. [CrossRef]