Macrophages That Survive Hyperoxia Exposure Have Higher Superoxide Dismutase Activities in Their Mitochondria

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Abstract Prolonged exposure to hyperoxia, which is routinely used in patients with severe respiratory failure, leads to the generation of excessive reactive oxygen species, resulting in lung injury. In the present study, we focused on macrophages and their survival, superoxide dismutase (SOD) activity in mitochondria (Mn-SOD activity), and mitochondrial DNA (mtDNA) mutation after exposure to hyperoxia. Macrophages were cultured under two different conditions: normoxia and intermittent hyperoxia. The number of cells exposed to intermittent hyperoxia for 3 weeks significantly decreased, compared with the number of cells exposed to normoxia. The Mn-SOD activity of the cells that survived intermittent hyperoxia exposure was significantly higher than that of the cells exposed to normoxia. Direct sequencing and a PCR-RFLP assay did not provide any evidence of mutation in the cells that survived intermittent hyperoxia exposure. In conclusion, an increase in the antioxidative activity of mitochondria is important for the survival of macrophages exposed to hyperoxia, and the increased activity level possibly enhances protective effects against mtDNA mutations in surviving cells.

1 Introduction

Prolonged exposure to hyperoxia, which is routinely used in patients with severe respiratory failure, leads to the generation of excessive reactive oxygen species (ROS) in the lungs, resulting in lung injury [1]. In the present study, we focused on macrophages, since alveolar macrophages are exposed to hyperoxia and their functions are important in host defense. We investigated their survival, the superoxide dismutase (SOD) activity in mitochondria (Mn-SOD activity), and mitochondrial DNA (mtDNA) mutation after exposure to hyperoxia.

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The major function of mitochondria is to produce energy to support cellular activities through the oxidative phosphorylation pathway [2]. During this process, ROS are generated. Since mtDNA is an easy target for oxidative DNA damage by ROS, the mutation rate of mtDNA is considered to be at least ten times higher than that of nuclear DNA [3, 4]. Therefore, the survival of macrophages exposed to hyperoxia might be related to their SOD activity and its protective effects against mtDNA mutation.

The aim of this study was to determine whether the Mn-SOD activity of surviving cells was altered and whether mtDNA mutations occurred after exposure to hyperoxia. To this end, we measured the changes in the number of cells and the Mn-SOD activities of cells exposed to hyperoxia and then analyzed mtDNA mutations in these cells using direct sequencing and a PCR-RFLP assay.

2 Materials and Methods

2.1 Macrophages Culture

We used macrophages that differentiated from a human monocyte cell line (THP-1, ATCC) after the cell line was cultured with phorbol myristate acetate for 2 days. The haplogroup of the mitochondria in the cells was classified as F1b1a. The cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (CELLect^{TM}; MP Biomedicals), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (GIBCO^{TM}; Invitrogen) at 37°C in a humidified 5% CO_2 atmosphere. The macrophages were cultured under two different conditions: normoxia (21% O_2 + 5% CO_2, for 3 weeks) and intermittent hyperoxia (three repeats of 90% O_2 + 5% CO_2 for 4 days and 21% O_2 + 5% CO_2 for 3 days; i.e., 3 weeks in total). Intermittent hyperoxia was applied in the present study because cells that were continuously exposed to hyperoxia all died within 2 weeks. Intermittent hyperoxia enabled a long-term experiment to be conducted, with the normoxia phases allowing the mitochondria to recover, grow, and possibly accumulate mutated mtDNA.

2.2 Cell Counting and SOD Activity Assay

We measured the changes in the number of cells and Mn-SOD activity, which reflects the antioxidative activity in mitochondria. The number of cells was determined using a counting chamber. Mn-SOD activity was measured using the SOD assay kit (Dojindo Laboratories) and KCN (final concentration: 40 μM) as an inhibitor of Cu/Zn-SOD [5].

2.3 Isolation and Amplification of DNA

Total DNA was extracted from the cells using the QIAamp Blood Mini Kit (QIAGEN). The entire mitochondrial genome was amplified as six fragments (3000–3400 bp) by the first PCR and 60 overlapping segments (600–1000 bp) by the second PCR. The primer pairs and their nucleotide sequences were described previously [6]. The primer pairs were designed to cover the entire mtDNA including an overlapped region that enabled us to confirm the sequences near the 3′- and 5′-ends of the PCR products using two fragments. The conditions for the first and second PCR were the same: 94°C for 5 min; 15 s at 94°C, 15 s at 60°C, 3 min at 72°C; and a final extension for 10 min at 72°C. The amplified fragments were analyzed using electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. These second PCR products were purified using MultiScreen-PCR Plates (Millipore). The quality of the DNA templates was examined using electrophoresis on a 1.2% agarose gel after staining with ethidium bromide using a Ready-To-Run Separation Unit (Amersham Pharmacia Biotech).

2.4 Sequence Analysis of Mitochondrial DNA

We analyzed the mutations in the mtDNA of the cells using direct sequencing after the cells had been exposed to intermittent hyperoxia or normoxia for 3 weeks. Sequence reactions were performed using a BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems). After excess dye terminators had been removed with MultiScreen-HV plates (Millipore) packed with Sephadex G50 superfine (Pharmacia), the purified DNA samples were precipitated with ethanol, dried, and suspended in the template suppression reagent or formamide (Applied Biosystems). The dissolved DNA samples were heated for 2 min at 95°C for denaturation, then immediately cooled on ice. The sequences were analyzed with automated DNA sequencers 377 and 310 using the Sequencing Analysis Program version 4.1 (Applied Biosystems).

2.5 PCR-RFLP Assay

We analyzed three suspected mutation sites in cells exposed to normoxia using a PCR-RFLP assay. These sites showed different direct sequencing results for the overlapping segment of the two different fragments in cells exposed to normoxia (see the Results section). Firstly, PCR was performed using 5 ng of the cDNA as a template and PCR buffer, dNTP mix, forward and reverse primers (5′-AAACCCT CGTTCCACAGAAG-3′ (forward primer) and 5′-TGATT-GAGATGGGGGCTA GT-3′ (reverse primer) for mt.4732, 5′-GTTCTTTCA TGGGGAAGCAG-3′ (forward primer) and 5′-GTGGCTTTGGAGTTG

CAGTT-3′ (reverse primer) for mt.16129, and 5′-CTGTTCTTTC ATGGG-GAAGC-3′ (forward primer) and 5′- TAGTTGAGGGTTGATTGCTG-3′ (reverse primer) for mt. mt.16189), and rTaq DNA polymerase (Toyobo). The reaction conditions were as follows: 94°C for 5 min; 15 s at 94°C, 15 s at 58–60°C (59.9°C for mt.4732, 59.3°C for mt.16129, 58°C for mt.16189), 3 min at 72°C for 40 cycles; and a final extension for 10 min at 72°C. The amplified fragments were analyzed using electrophoresis on a 1% agarose gel and visualized by staining with ethidium bromide. Then, restriction enzyme was added to each fragment solution. We used Sca I [10 U/μL]

(Roche), BsrG I [10 U/μL] (Daiichi Pure Chemicals), or Mnl I [2 U/μL] (Daiichi Pure Chemicals), incubated with the PCR products at 37°C for 1 h to cut the sequences of AGTACT, TGTACA, and CCTC, respectively. Electrophoresis on a 3% agarose gel was performed to detect the fragment size after digestion with the restriction enzyme.

2.6 Statistical Analysis

All data are given as the mean ± SD. Statistically significant differences were determined using a Wilcoxon matched-pair signed-rank test (cell counting assay) or a Student *t*-test (SOD assay). A probability value (p-value) of less than 0.05 was considered statistically significant.

3 Results

The number of cells exposed to intermittent hyperoxia for more than a week significantly decreased, compared with the number of cells exposed to normoxia (Fig. 1a). The Mn-SOD activity per 100 cells increased with time and was significantly higher in the cells exposed to intermittent hyperoxia than in the

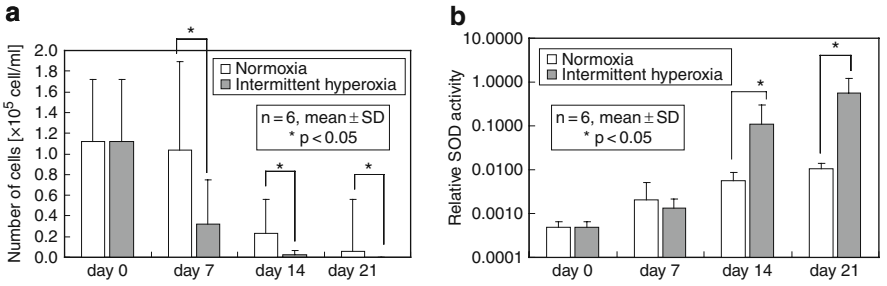

Fig. 1 (**a**) Changes in the number of cells exposed to normoxia or intermittent hyperoxia. (**b**) Relative SOD activity per 100 macrophages. Relative SOD activity was defined based on a value at which 100 cells eliminated all the available superoxide

cells exposed to normoxia (Fig. 1b). The Mn-SOD activity of the surviving cells was about half of the total SOD activity (data not shown).

Using direct sequencing, six sites (nucleotide position: mt.4732, mt.16129, mt.16189, mt.16232, mt.16249, mt.16362) in the mtDNA were identified as suspected sites of mutation located in one coding region and five non-coding regions of the D-loop in mtDNA (Fig. 2). These six suspected sites were located in the overlapped region of the alternative fragments in the second PCR amplification. At these sites, a heteroplasmic sequence was observed in the cells exposed to normoxia. For example, sequence chromatogram showed overlapped peaks of A and G, suggestive of heteroplasmy, at nucleotide position 4732 in the fragment 16. Analysis of the neighboring second PCR product (fragment 17), however, showed only a peak of C at the same site, suggestive of homoplasmy. To confirm this result, Three of the six sites, including the one in a coding region (mt.4732), were amplified by one-step direct amplification from DNA isolated from the cells and were further verified using a PCR-RFLP assay for these potentially heteroplasmic sites. The PCR products were almost completely digested by the restriction enzyme or were completely undigested (Fig. 2), meaning that these three sites were not heteroplasmic and were not mutation sites. We confirmed that complete digestion was attained using another cDNA with the same recognition sites as a positive control (data not shown).

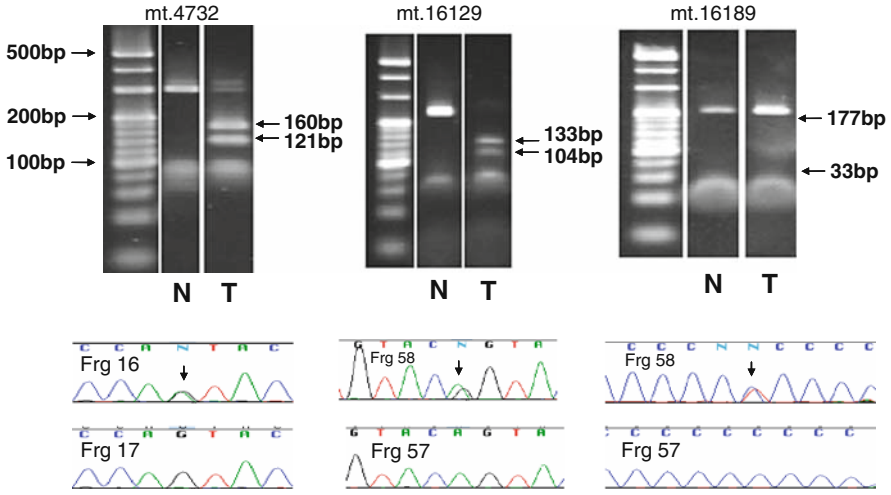

Fig. 2 Gel electrophoresis of PCR-RFLP products and gene sequences measured using direct sequencing of cells exposed to normoxia. N: PCR products without treatment, T: PCR products treated with restriction enzyme. The PCR products, including mt.4732 and mt.16129, were almost completely digested by the restriction enzyme, while mt.16189 was completely undigested (no bands observed at 177 and 33 bp), meaning that these three sites were not heteroplasmic, although a heteroplasmic sequence was suggested by the alternative fragments in the direct sequencing of the other DNA fragments amplified with another pair of primers

4 Discussion

In the present study, we found that hyperoxia induced the cell death of macrophages and that the macrophages that survived intermittent hyperoxia exposure had higher Mn-SOD activities and no mutations in their mtDNA. The survival of the macrophages exposed to intermittent hyperoxia was related to their SOD activities and possibly to their protective effects against mtDNA mutations induced by oxidative DNA damage. Cells that were exposed to hyperoxia but could not increase their SOD activity sufficiently might have been killed by the resulting oxidative DNA damage.

Because of the lack of protective histone proteins and a limited DNA repair mechanism, mtDNA is more susceptible to oxidative DNA damage [3, 4] than nuclear DNA. In the present study, the Cu/Zn-SOD activity of the surviving cells, which reflects the SOD activity in the cytosol, increased as well as the Mn-SOD activity (data not shown), suggesting that the nuclear DNA was also protected from the oxidative stress. Therefore, the possibility of oxidative damage to the nuclear DNA in the surviving macrophages after hyperoxia exposure was considered to be relatively low. Our results implied that antioxidative activities in the cytosol and mitochondria are important and can enhance the survival of macrophages exposed to hyperoxia.

A PCR-RFLP assay was considered to be a more reliable assay to detect mutations than direct sequencing, showing no heteroplasmic sequences, indicating that the three sites were not mutation sites. Although not all of the suspected mutation sites were verified using a PCR-RFLP assay because of the limited availability of appropriate restriction enzymes, the three suspected mutation sites that were not examined were located in non-coding regions, which would have produced innocent mutations even if they had occurred. Thus, no mutations in coding regions occurred in the surviving cells, and any cells with fatal mutations likely died after the hyperoxic insult.

In conclusion, the increase in antioxidative activity in mitochondria is important for the survival of macrophages exposed to hyperoxia, and this increase possibly enhances protective effects against mtDNA mutation in surviving cells.

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References

1. Zaher TE, Miller EJ, Morrow DM et al. (2007) Hyperoxia-induced signal transduction pathways in pulmonary epithelial cells. Free Radic Biol Med 42:897–908.
2. Attardi G, Schatz G (1988) Biogenesis of mitochondria. Annu Rev Cell Biol 4:289–333.
3. Wallace DC, Shoffner JM, Trounce I et al. (1995) Mitochondrial DNA mutations in human degenerative diseases and aging. Biochim Biophys Acta 1271:141–151.

4. Copeland WC, Wachsman JT, Johnson FM et al. (2002) Mitochondrial DNA alterations in cancer. Cancer Invest 20:557–569.
5. Oyanagui Y (1984) Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. Anal Biochem 142:290–296.
6. Tanaka M, Hayakawa M, Ozawa T (1996) Automated sequencing of mitochondrial DNA. Methods Enzymol 264:407–421.