Enhanced expression of nicotinamide nucleotide transhydrogenase (NNT) and its role in a human T cell line continuously exposed to asbestos

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1. Introduction

Exposure to asbestos fibers can cause asbestosis, a typical form of pneumoconiosis, and which is characterized as lung fibrosis (Wolff et al., 2015; Furuuya et al., 2018). Furthermore, there are many asbestos-related diseases such as pleural plaque (PP), diffuse pleural thickening and benign pleural effusion which represent non-malignant diseases (Lazarus et al., 2012). Additionally, asbestos can cause lung cancers, malignant mesothelioma (MM) and other cancers (Lazarus et al., 2012; Stayner et al., 2013; Prazakova et al., 2014). The most important factor pertaining to asbestos carcinogenesis is iron (Kamp, 2009; Toyokuni, 2019). The iron generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) via Fenton’s reaction at inhaled and remaining sites such as the lung, pleural cavity and regional lymph nodes (Kamp, 2009; Toyokuni, 2019).

The immunological effects of asbestos are not yet fully investigated. Regarding T helper (Th) cells, we have utilized the human T-lymphotropic virus 1 (HTLV-1)-immortalized human T cell line MT-2. Sublines continuously exposed to chrysotile (CH) or crocidolite (CR) showed acquired resistance to asbestos-induced apoptosis following transient and high-dose re-exposure with fibers. These sublines in addition to other immune cells such as natural killer cells or cytotoxic T lymphocytes exposed to asbestos showed a reduction in anti-tumor immunity. In this study, the expression of genes and molecules related to antioxidative stress was examined. Furthermore, complexes related to oxidative phosphorylation were investigated since the production of reactive oxygen species (ROS) is important when considering the effects of asbestos in carcinogenesis and the mechanisms involved in resistance to asbestos-induced apoptosis. In sublines continuously exposed to CH or CR, the expression of thioredoxin decreased. Interestingly, nicotinamide nucleotide transhydrogenase (NNT) expression was markedly enhanced. Thus, knockdown of NNT was then performed. Although the knockdown clones did not show any changes in proliferation or occurrence of apoptosis, these clones showed recovery of ROS production with returning NADPH/NADP+ ratio that increased with decreased production of ROS in continuously exposed sublines. These results indicated that NNT is a key factor in preventing ROS-induced cytotoxicity in T cells continuously exposed to asbestos. Considering that these sublines showed a reduction in anti-tumor immunity, modification of NNT may contribute to recovery of the anti-tumor effects in asbestos-exposed T cells.

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ABSTRACT

The effects of asbestos fibers on human immune cells have not been well documented. We have developed a continuously exposed cell line model using the human T-lymphotropic virus 1 (HTLV-1)-immortalized human T cell line MT-2. Sublines continuously exposed to chrysotile (CH) or crocidolite (CR) showed acquired resistance to asbestos-induced apoptosis following transient and high-dose re-exposure with fibers. These sublines in addition to other immune cells such as natural killer cells or cytotoxic T lymphocytes exposed to asbestos showed a reduction in anti-tumor immunity. In this study, the expression of genes and molecules related to antioxidative stress was examined. Furthermore, complexes related to oxidative phosphorylation were investigated since the production of reactive oxygen species (ROS) is important when considering the effects of asbestos in carcinogenesis and the mechanisms involved in resistance to asbestos-induced apoptosis. In sublines continuously exposed to CH or CR, the expression of thioredoxin decreased. Interestingly, nicotinamide nucleotide transhydrogenase (NNT) expression was markedly enhanced. Thus, knockdown of NNT was then performed. Although the knockdown clones did not show any changes in proliferation or occurrence of apoptosis, these clones showed recovery of ROS production with returning NADPH/NADP+ ratio that increased with decreased production of ROS in continuously exposed sublines. These results indicated that NNT is a key factor in preventing ROS-induced cytotoxicity in T cells continuously exposed to asbestos. Considering that these sublines showed a reduction in anti-tumor immunity, modification of NNT may contribute to recovery of the anti-tumor effects in asbestos-exposed T cells.
contaminated iron content and other elements (Kohyama et al., 1996), and CH-A is mined and obtained from Zimbabwe while CH-B is from Canada) or to CR at relatively low doses (where less than half the number of MT-2Org cells proceeded to apoptosis) (Hyodoh et al., 2005; Miura et al., 2006; Maeda et al., 2012). We found that these sublines (designated CB1 to CB3 for cells exposed to CH-B, CA1 to CA-3 for cells exposed to CH-A, and CR1 to CR3 for cells exposed to CR) showed similar cellular and molecular alterations such as production of cytokotins characterized by over-production of interleukin (IL)-10 and transforming growth factor (TGF)-β, and reduced production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Hyodoh et al., 2005; Miura et al., 2006; Maeda et al., 2012). Additionally, these sublines showed reduced cell surface C-X-C motif chemokine receptor 3 (CXCR3) (Maeda et al., 2011a, 2011b). Since CXCR3-expressing Th cells summon tumor attacking T cells to nearby tumor cells, these alterations indicated that exposure to asbestos produces a reduction in anti-tumor immunity. Moreover, since MT-2Org cells were reported to possess regulatory T (Treg) cell function (Chen et al., 2006; Hamano et al., 2015), we examined Treg cell function in MT-2Org cells unexposed to fibers and in sublines continuously exposed to fibers. The study showed that asbestos enhanced Treg cell function by cell-to-cell contact and increased the production of soluble factors (as mentioned above, sublines of MT-2Org which exposed continuously to asbestos showed increased production of IL-10 and TGF-β) (Ying et al., 2015). Moreover, lower levels of FoxO1, a transcription factor, in sublines compared to MT-2org, resulted in the acceleration of cell cycle progression by increasing cyclins and decreasing cyclin-dependent kinase inhibitors (CDKs) such as the ink4 and CIP/KIP families (Lee et al., 2017a). Additionally, decreased FoxO1 resulted in a reduction in various pro-apoptotic factors such as fas ligand, bim and puma (Matsuzaki et al., 2016). In fact, these sublines revealed resistance to asbestos-induced apoptosis via decreased cell death signals regulated by FoxO1 and increased Bcl-2 via enhanced phosphorylation of signal transducers and activator of transcription (STAT) 3 caused by autocrine utilization of over-produced IL-10 (Miura et al., 2006). All of these findings indicated that asbestos exposure causes a reduction in anti-tumor immunity, which results in aggressive development of asbestos-induced cancers after a long-term latency period of 30–40 years (Kumagai-Takei, et al.; 2011, 2018; Matsuzaki et al., 2012). These findings are summarized in Fig. 1. As mentioned above, the low-dose were decided by doses which induced less than half of apoptosis in MT-2Org cells by transient exposure. Although the exact comparison with human exposure doses including doses exposed to immune cells located in pulmonary and pleural regions were not done, the changes in these sublines were confirmed by asbestos exposed patients such as PP and MM (Miura, et al: 2006). Thus, we assumed these experimental exposed low-doses may reflect cellular phenomenon occurred in asbestos exposed people.

As mentioned above, transient and relatively high-dose exposure of MT-2Org cells to CH or CR led to the production of ROS (Hyodoh et al., 2005; Maeda et al., 2012). Continuously exposed sublines (CB1 to CB3, CA1 to CA3, and CR1 to CR3) showed resistance to asbestos-induced apoptosis and a lower production of ROS (Maeda et al., 2012). Thus, the expression pattern of enzymes related to antioxidative stress and redox reactions may be altered in continuously exposed sublines. Furthermore, if sublines can effectively render harmless the ROS produced following contact with asbestos, certain alterations in the oxidative phosphorylation (OXPHOS) complex may occur in sublines. The OXPHOS complex comprises five multimeric enzymes located on the mitochondrial membrane (Rydstrom 2006; Papa et al., 2012; Ciccarese and Ciminale, 2017; Maio et al., 2017; Salazar, 2018). Complex I comprises nicotinamide adenine dinucleotide (NADH)-ubiquinone reductase (H+-translocating), complex II comprises succinate dehydrogenase (SDH), complexes III and IV comprise ubiquinol-cytochrome-c (Cyt-C) reductase and cytochrome c oxidase (COX), respectively, while complex V comprises adenosine triphosphate (ATP) synthase. The OXPHOS system, comprised of five multimeric enzyme complexes, drives the synthesis of ATP in most cells. Cyt-C represents one of the mobile carriers. Complexes I to IV transport H+ to the intermembrane space of mitochondria. Complex V, an ATPase, transports H+ from the intermembrane space to the inside of mitochondria to generate adenosine triphosphate (ATP) as an energy source. Furthermore, nicotinamide nucleotide transhydrogenase (NNT) also transports H+ from the intermembrane space to the inside (Rydstrom 2006; Ho et al., 2017). With this reaction, an oxidized form of NAD phosphate (NADP +) is converted to the reduced form of NADP (NADPH). The repeated redox reaction between NADP+ and NADPH also converts glutathione disulfide (GSSG) to glutathione (GSH). The GSSG/GSH redox cycle consumes superoxide captured by superoxide dismutase (SOD) 2 and is utilized to prevent DNA damage caused by ROS (Rydstrom 2006; Papa et al., 2012; Ciccarese and Ciminale, 2017; Ho et al., 2017; Maio et al., 2017; Salazar, 2018).

Thus, in this study, alterations in the expression of enzymes related to antioxidative stress and redox reactions, as well as expression of OXPHOS components, were examined.

2. Materials and methods

2.1. Cell lines

An MT-2 cell line and sublines CB1 and CR1 were used in this study. The MT-2Org was established as previously reported (Miyoishi et al., 1981a, 1981b). The establishment and some analyses of continuously exposed CH-B and CR sublines have been previously reported (Hyodoh et al., 2005; Miura et al., 2006; Maeda et al., 2012). MT-2org and sublines CB1 and CR1 were maintained in a humidified atmosphere of 5% CO2 at 37 °C in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), streptomycin and penicillin. For western blot analyses and expression analyses of the OXPHOS complex with NNT, CB1 and CR1 sublines continuously exposed to CH and CR asbestos fibers, respectively, were then subjected to Ficoll-Hypaque gradient centrifugation to remove the fibers. Both sublines collected from this procedure were then cultured for one week in the absence of fibers. Thereafter, MT-2org and fiber-removed CB1 and CR1 sublines were exposed to 50, 100 and 200 μg/ml CH or CR (sublines CB1 and CR1 exposed only to CH and CR, respectively). Cells were then collected for analyses.

2.2. NNT knockdown clones

To obtain NNT knockdown stable clones derived from the CB1 subline, lentiviral transduction was performed. The target double-stranded oligonucleotide sequences employed for the NNT gene comprised (1) for the KD4 clones; CCGGGCCACCTTTGGTGAGATTTCT CGGAGATAATCCACAAAGGTTGGTTTG (2) for the KD5 clones; CCGGACCTATGGTTAATCCAACATCTCGGAGATTTGTTAGGATTA ACCATAAGGTTTTG. These targets were selected from the MISSION shRNA clone (Sigma-Aldrich Japan, Co. LLC. Tokyo, Japan) designated as # SHCLND. The lentiviral vector employed was pLKO.1-puro (Addgene Ltd., Watertown, MA) The control vector employed was Scramble shRNA (Addgene) Cat. No. 1864. The oligonucleotides were subcloned into lentiviral vector pLKO.1-puro as previously described (Naldini et al., 1996). The vesicular stomatitis virus G protein (VSV-G)- pseudotyped HIV-1-based vector system was generated as previously described (Zufferey et al., 1997; Bridge et al., 2003). The replication-defective lentiviral vector particles were produced by transient co-transfection of Lentiviral Packaging Mix including pLP1, pLP2 and pLP3/vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1-based vector system was generated as previously described (Zufferey et al., 1997; Bridge et al., 2003). The replication-defective lentiviral vector particles were produced by transient co-transfection of Lentiviral Packaging Mix including pLP1, pLP2 and pLP3/vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1-based vector system was generated as previously described (Zufferey et al., 1997; Bridge et al., 2003).
puromycin to select stable clones expressing shRNA.

### 2.3. Asbestos fibers

The International Union Against Cancer standard CH-A and CH-B were kindly provided by the Department of Occupational Health at the National Institute for Occupational Health of South Africa. The mineralogical features of these fibers have been previously reported. Additionally, CH-B and CR were also provided by the Japan Association for the Study of Fiber Materials as standard samples only for experimental use.

### 2.4. Real-time PCR

Total cellular mRNA from MT-2Org, CB1 and CR1 cells was extracted using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Following synthesis of the first strand cDNA, real-time RT-PCR was performed using the SYBR Green method (TaKaRa Bio Inc., Kusatsu, Japan) with the Mx3000P QPCR System (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer’s instructions. The primers used are shown in Table 1.

### 2.5. Western blot analyses

Proteins from whole cells of the MT-2Org line and CB1 and CR1 sublines as well as NNT knockdown clones designated KD4 and KD5 derived from CB1 were extracted using Pierce® RIPA Buffer (Thermo Fisher Scientific Inc., Cat. No. 89901) according to the manufacturer’s instructions. For NNT expression, proteins from mitochondria and the cytosol were extracted. Fractionation was performed using an ApoAlert® Cell Fractionation Kit (Clontech Laboratories, Inc. [presently TaKaRa Bio USA, Inc.], Mountain View, CA., Cat. No. 630105) according to the manufacturer’s instructions.

Western blot analyses were performed as described in previous reports (Lee et al., 2017a). Proteins were probed with the following antibodies: anti-NNT (Sigma-Aldrich, Cat. No. HPA004829), anti-cytochrome c oxidase subunit 4 (COX4) (Cell Signaling Technology, Danvers, MA., Cat. No. 11967), anti-superoxide dismutase 2 (SOD2) (Cell Signaling Technology, Cat. No. 13194), anti-catalase (Cell Signaling Technology, Cat. No. 14097), anti-peroxiredoxin 3 (PRDX3) (Cell Signaling Technology, Cat. No. 8499), anti-thioredoxin2 (Cell Signaling Technology, Cat. No. 14907), anti-heat shock protein (HSP) 60 (Cell Signaling Technology, Cat. No. 12165), anti-glutaredoxin (GLRX2) (BosterBio Ltd., Pleasanton, CA., Cat. No. PA1885) and anti-peroxiredoxin3 (Bethyl Laboratories, Ltd., Montgomery, TX., Cat. No. A304-744A-T). Proteins were then incubated with HRP-conjugated anti-mouse IgG or anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected using ECL Plus Western Blotting Detection Reagents (GE Healthcare UK Ltd., Buckinghamshire, England).

For the separation of cytosolic and mitochondrial fractions, an ApoAlert® Cell Fractionation Kit (Clontech, now supplied by TaKaRa Bio, Cat. No. 630105) was used according to the manufacturer’s instructions.

### 2.6. Measurement of mitochondria

The amount of intracellular mitochondria in MT-2Org, CB1 and CR1 cells was measured by employing MitoTracker (Thermo Fisher Scientific, Cat. No. M7514) with fluorescence microscopy and flow cytometry (FACScan II System; Becton Dickinson Bioscience, Ltd., Franklin Lakes, NJ) according to the manufacturer’s instructions.

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**Fig. 1.** Schematic presentation of our established cell line model comprising continuous exposure to asbestos of the human T-lymphotropic virus 1 (HTLV-1)-immortalized human T cell line MT-2 (MT-2Org). When MT-2Org cells were exposed to chrysotile or crocidolite asbestos fibers, cells proceeded to apoptosis with production of reactive oxygen species (ROS), where the amount of ROS was higher with crocidolite exposure compared to chrysotile exposure. However, relatively low dose exposure (did not induce apoptosis in less than half the number of MT-2Org cells) and continuously exposed sublines (designated as CB1 to CB3 for chrysotile exposure, and CR1 to CR3 for crocidolite exposure) showed resistance to asbestos-induced apoptosis when cells were exposed to high doses of fibers. These sublines showed overproduction of IL-10 and TGF-β, and reduced CXCR3 and IFN-γ and TNF-α production. Furthermore, since MT-2 was reported to possess a regulatory T cell (Treg) phenotype, the function and proliferation of sublines such as Treg cells were examined. Results showed that Treg cell function was enhanced and that the cell cycle was accelerated in sublines. These findings indicated that continuous and relatively low-dose exposure of immune cells to asbestos resulted in a reduction in anti-tumor immunity.
Table 1
Primer sequences for RT-PCR.

| Gene   | Forward                  | Reverse                   |
|--------|--------------------------|---------------------------|
| CAT    | 5′-GAGCCTAGTGTCTGAGTTC-3′ | 5′-GGACGTTCTGAGGACGGTT-3′ |
| GLRX   | 5′-GTAATAGCCCTGGAAGCAG-3′ | 5′-GGAAGAACCCCTGAGGAT-3′ |
| GPX1   | 5′-AATCCCGAAGCAGTGCTCT-3′ | 5′-GGAGAGATGAGAATGGTG-3′ |
| PRDX1  | 5′-AAGCTTTCTGAGGACGGTA-3′ | 5′-GCAATTTGAGGACGGTT-3′ |
| PRDX3  | 5′-GGAGATGATGAGAATGGTG-3′ | 5′-GGAGAGATGAGAATGGTG-3′ |
| SOD1   | 5′-CAGGATGAAATGTGTGCT-3′  | 5′-GCAATTTGAGGACGGTT-3′ |
| SOD2   | 5′-ATGTGTGAGGACGGTT-3′    | 5′-GCAATTTGAGGACGGTT-3′ |
| SOD3   | 5′-ATGTGTGAGGACGGTT-3′    | 5′-GCAATTTGAGGACGGTT-3′ |
| TXN    | 5′-GTAATAGCCCTGGAAGCAG-3′ | 5′-GGAAGAACCCCTGAGGAT-3′ |
| NNT    | 5′-GGAAGAACCCCTGAGGAT-3′  | 5′-GGAAGAACCCCTGAGGAT-3′ |
| GAPDH  | 5′-GGAAGAACCCCTGAGGAT-3′  | 5′-GGAAGAACCCCTGAGGAT-3′ |

The abbreviations are CAT; Catalase, GLRX; Glutaredoxin, GPX1; Glutathione peroxidase 1, PRDX1; Peroxiredoxin 1, PRDX3; Thioredoxin-dependent peroxide reductase/ Peroxiredoxin 3, SOD1; Superoxide dismutase 1, SOD2; Superoxide dismutase 2, SOD3; Superoxide dismutase 3, TXN; Thioredoxin, NNT; Nicotinamide nucleotide transhydrogenase, and GAPDH; Glyceraldehyde-3-phosphate dehydrogenase.

Fig. 2. Western blot analyses of MT-2Org (Org), CB1, CR1 cells and samples exposed to high doses of asbestos fibers for 24 h: 50 μg/ml chrysotile (CH) or 100 μg/ml crocidolite (CR) for MT-2Org, chrysotile for CB1, and crocidolite for CR1. Asbestos fiber doses were determined from our previous reports. (A) Representative Western blot images. Panels B to F showed averages and standard deviations (SD) obtained from three independent experiments: catalase (B), superoxide dismutase (SOD) 2 (C), peroxiredoxin (PRDX) 3 (D), thioredoxin 2 (E), and glutaredoxin (GLRX) 2 (F). There was no statistical significance when analyzed by Mann-Whitney U test.
2.7. Multiplex analysis of OXPHOS complex and NNT expression

Cells (3 × 10^6) from MT-2Org, CB1 and CR1 (both sublines had fibers removed one week previously) were cultured in 6-well plates with 50 μg/ml CH fibers for MT-2Org and CB1, or 100 μg/ml CR fibers for MT-2Org and CR1 for 3 days. The doses were determined from our previous reports. Cells were then collected and assayed for OXPHOS complex and NNT expression using a MILLIPLEX MAP Human Oxidative Phosphorylation (OXPHOS) Magnetic Bead Panel-Cellular Metabolism Multiplex Assay (Merck Millipore Inc., Burlington, MA, Cat. No. H0XPSMAG-16 K) and a Luminex 200 System (Luminex® 200 × PONENT® System, Merck Millipore) according to the manufacturer’s instructions.

2.8. Assay for cell growth

The assay for cell growth was monitored using a Premix WST-1 Cell Proliferation Assay System (Takara Bio Inc., Tokyo, Japan, Cat. No. MK400). Cells (2 × 10^4) from MT-2Org, CB1, NNT knockdown clone #5 (KD5), and CB-transfected scrambled oligonucleotide for knockdown (Scr) as a control were cultured in 96-well plates in the absence or presence (200 μg/ml) of CH fibers. Following 48 h incubation, cell growth was assessed by measuring the absorbance using a microplate reader according to the manufacturer’s instructions.

2.9. Analysis of apoptosis by Annexin V staining.

MT-2Org, CB1, KD5 and Scr cells (1 × 10^5 cells/well) were cultured in the absence or presence (200 μg/ml) of CH fibers in 24-well plates for 24 h. Apoptotic cells were detected by staining with Annexin-V-FITC (Sigma Aldrich, Cat. No. 1858777) and 7-Amino-Actinomycin D (7-AAD) (BD Biosciences, Cat. No. 559925) according to the manufacturer’s protocol, and stained cells were analyzed using a flow cytometer (FACSCanto II System, BD Biosciences).

2.10. Detection of ROS

MT-2Org, CB1, NNT knockdown clone #4 (KD4), and Scr cells

Fig. 3. The results of three independent experiments utilizing real-time RT-PCR for the investigation of enzymes related to antioxidative stress and redox reactions using MT-2Org, CB1 and CR1 sublines. Fibers in subline cultures were removed one week previously by gradient centrifugation. There was no statistical significance when analyzed by Mann-Whitney U test.
(1 × 10⁵/ml) were cultured in the absence or presence (50, 100 and 200 μg/ml) of CH fibers in 24-well plates for 18 hr. Following incubation, ROS in the cells were stained using Image-IT™ LIVE Green Reactive Oxygen Species Detection Kit (Thermo Fisher Scientific, Cat. No. I36007) and then analyzed by flow cytometry (FACSCanto II System) according to the manufacturer’s instructions.

Furthermore, CB1 and CR1 cells (1 × 10⁵ cells/well) were cultured in 24-well plates in the absence or presence of asbestos fibers (CH for CB1, and CR for CR1, 100 μg/ml each) for 4 h with NNT inhibitor (Palmitoyl Coenzyme A Potassium Salt, Nacalai Tesque, Inc., Kyoto, Japan, Cat. No. 25920–64) and control (Kcl) according to the manufacturer’s instructions. ROS production was then assayed using a FACSCant II System.

2.11. Measurement of nicotinamide adenine dinucleotide phosphate (NADP)+ and reduced form of NADP (NADPH)

MT-2Org, CB1 and KD5 cells (2 × 10⁶ cells/well) were cultured in 6 cm dishes for 18 h in the absence or presence (50 μg/ml) of CH fibers. The amounts of NADP+ and NADPH were then measured using an NADP/NADPH Quantification Colorimetric Kit (BioVision Inc., Milpitas, CA., Cat. No. K347-100) according to the manufacturer’s instructions. The ratio of NADPH/NADP+ was also calculated.

2.12. Measurement of oxidized glutathione (glutathione-S-S-glutathione; GSSG) and reduced glutathione (GSH)

MT-2Org, CB1 and KD5 cells (2 × 10⁶ cells/well) were collected from cultures, washed with phosphate-buffered saline (PBS), and then treated with Hcl. After two freeze-thaw cycles, 5% S-sulfosalicylic acid was added to the samples. GSSG and GSH were then measured using a GSSG/GSH Quantification Kit (Dojindo, Cat. No. G257) according to the manufacturer’s instructions.

2.13. Statistical analysis

For the all experiments, the experimental number were ranged 3–5. Statistical analyses were performed using SPSS version 21 (IBM Japan, Tokyo, Japan). Protein, mRNA expression, flow cytometry analysis of OXPHOS complex, NNT expression in mitochondria, growth properties assayed by WST-1, appearance of apoptotic cells assayed by Annexin V, flow cytometry analysis of ROS production and ratios of NADPH/NADP+ and GSSG/GSH were compared among MT-2Org, CB1, CR1 and NNT knockdown clones (KD4 or KD5, including control shRNA clone (Scr)) cells, including cells untreated or treated with asbestos fibers. Mann-Whitney U test, Bonferroni test, and unpaired student T-test were used to determine the statistical significance of differences among
3. Results

3.1. Protein expression in MT-2Org and continuously exposed sublines and cells exposed or re-exposed to high-dose fibers

Fig. 2 shows a comparison of proteins related to antioxidative stress. Whole-cell proteins were extracted from MT-2Org, CB1 and CR1 sublines (continuously exposed to fibers, CH for CB1 and CR for CR1 after fiber removal for one week). Additionally, proteins were extracted from MT-2Org and fiber-removed CB1 lines exposed to 50 μg/ml CH fibers for two days, and MT-2Org and fiber-removed CR1 lines exposed to 100 μg/ml CR fibers for two days. These fiber concentrations have been reported to cause apoptosis in MT-2Org cells.

Fig. 2A shows blot images and comparisons are shown in Fig. 2B-F (2B; catalase, 2C; SOD1, 2D; PRDX1 and 2E; Thioredoxin2 and 2F; GLRX2). HSP60 was used as a control. Basically, there are no statistical significances among all the protein expression levels assayed here. Taken together, no pattern was observed in these molecules when comparing MT-2Org and continuously exposed sublines or MT-2Org and sublines subjected to high-dose fiber exposure.

3.2. mRNA expression of genes related to antioxidative stress in MT-2Org and sublines

Messenger RNA expression of genes related to antioxidative stress such as catalase, GLRX, glutathione peroxidase 1 (GPX1), PRDX 1 and 3, SOD 1, 2 and 3 and thioredoxin in MT-2Org cells and continuously exposed sublines (CB1 and CR1) was examined. As shown in Fig. 3, although the expression of thioredoxin in CB1 and CR1 continuously exposed sublines c seemed to be reduced compared with MT-2Org, the expression of all genes among these cells did not differ, statistically. Taken together, examination of continuously exposed sublines then subjected to high-dose fiber exposure did not reveal any significant findings regarding resistance to asbestos-induced apoptosis.

3.3. Expression of OXPHOS complex and NNT

Expression of the OXPHOS complex and NNT were examined since oxidative phosphorylation is closely related with removal of ROS. The presence of asbestos fibers results in the production of ROS which activate the mitochondrial apoptotic pathway to facilitate cell death. However, it was found that CB1 and CR1 sublines continuously exposed to CH and CR fibers, respectively, acquired resistance to asbestos-induced apoptosis. Thus, some alteration in the OXPHOS complex may contribute towards the acquisition of apoptosis resistance.

The expression of OXPHOS complex components I to V and related NNT enzyme were analyzed by flow cytometry. The results are shown in Fig. 4A to F. Complex I expression was lower in CR1 re-exposed transiently higher doses when compared with MT-2Org exposed transiently the same dose of CR and CB1-exposed transiently by high doses of CB (Fig. 4A). For complex III, CB1 showed lower and CR1 showed higher expression compared with MT-2Org. Then, CB1 was lower than CR1. In addition, these findings were similar when these cells re-exposed transiently with higher doses of fibers (CB or CR) (Fig. 4C). Similarly, complex V expression was higher in CR1 compared to MT-2Org cells. Additionally, re-exposure of CR1 to CR fibers resulted in increased expression of complex V, unlike the case with CB1 where complex V expression remained unaltered following re-exposure to CB fibers (Fig. 4E). As before, these findings may be dependent on the physical and chemical differences of the fiber types. Furthermore, it was found that expression of complexes II (Fig. 4B) and IV (Fig. 4D) was not significantly altered between MT-2Org, CB1, CR1 and cells exposed or re-exposed to high-dose fibers.

Interestingly, NNT expression in CB1 and CR1 was markedly higher compared to MT-2Org cells. Even though re-exposure of CB1 and CR1 to CH and CR fibers, respectively, did not result in changes in NNT expression, expression of NNT was markedly enhanced in sublines continuously exposed to fibers, as shown in Fig. 4F. NNT is known to be involved in hydride transfer between NADP+ and NADPH. Consequently, NNT activity induces a high concentration of NADPH. The NADPH in mitochondria is utilized in the removal of ROS via the cycling of GSH to GSSG. Taken together, increased expression in CB1 and CR1 sublines continuously exposed to fibers may facilitate resistance to asbestos-induced apoptosis.

Following examination of the expression of proteins and mRNA related to antioxidative stress, the OXPHOS complex and NNT, our experimental focus shifted to NNT.

3.4. Amount of mitochondria

The amount of mitochondria in MT-2Org, and CB1 and CR1 sublines continuously exposed to CH and CR fibers, respectively, was examined. Representative results are shown in Fig. 5A, which indicated that sublines contained slightly fewer mitochondria compared to MT-2Org, as determined by mitochondrial fluorescence assays using MitoTracker® in immunohistochemical and flow cytometry examinations. However, as shown in Fig. 5B, there were no significant changes statistically in three
Bonferroni’s test indicating significance, and there was a significant correction value of COX4. Then, Shapiro-Wilk’s normality test showed normal distribution and Levene’s statistic had equal variance p = 0.063. One way ANOVA to mitochondria were not induced by continuous exposure to independent experiments. Thus, marked changes in the amount of mitochondria were not induced by continuous exposure to fibers.

3.5. NNT mRNA and protein expression in mitochondria and NNT knockdown (ND) in CB1

NNT mRNA expression among MT-2Org, CB1 and CR1 cells was examined to confirm the results obtained by flow cytometry. As shown in Fig. 6A, continuously exposed sublines showed significant upregulation of NNT. NNT protein expression in mitochondria (but not in the cytosolic fraction) (Fig. 6B) was also enhanced, as determined by a multiplex assay using Luminex. A graphical presentation of NNT expression assayed by Western blotting with three independent experiments is shown in Fig. 6C.

In an effort to examine the role of NNT in CB1 cells which possess resistance to asbestos-induced apoptosis and overexpression of NNT, NNT knockdown in CB1 cells was performed using a lentiviral vector. Western blotting confirmed NNT knockdown in KD4 or KD5 clones, and its absence in the scrambled oligonucleotide transfected clone (Scr), as shown in Fig. 6D and E.

3.6. Effects of NNT knockdown on CB1 proliferation and occurrence of resistance to asbestos-induced apoptosis

The effects of NNT knockdown in CB1 on cell proliferation and occurrence of asbestos-induced apoptosis were examined. As shown in Fig. 7A, cell proliferation was assayed using the WST-1 method. In MT-2Org cells, 200 μg/ml CH caused inhibition of proliferation. Although this CH dose also caused a slight reduction in proliferation of CB1 and the KD5 and Scr clones, the degree of growth inhibition in these three cell types was lower compared to MT-2Org. There was no significant difference among the CB1, KD5 and Scr cell types. Thus, NNT knockdown did not seem to have a marked effect on cell proliferation.

The occurrence of apoptosis under uniform culture conditions was assayed using the Annexin V method as shown in Fig. 7B and C. Here, early apoptotic fractions were monitored (designated as Q4 in Fig. 7B, left upper panel). As shown in Fig. 7C, the degree of apoptosis in the early apoptotic fractions were monitored (designated as Q4 in Fig. 7B, left upper panel). As shown in Fig. 7C, the degree of apoptosis in the CB1, KD5 and Scr clones, the degree of growth inhibition in these three cell types was lower compared to MT-2Org. There was no significant difference among the CB1, KD5 and Scr cell types. Thus, NNT knockdown did not seem to have a marked effect on cell proliferation.

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3.7. ROS production in NNT or NNT-inhibitor knockdowns in CB1 and CR1

Silencing NNT did not affect cell proliferation or the occurrence of apoptosis caused by asbestos fibers, and so the next area to be examined was ROS production. The production of ROS was assayed by flow cytometry. As shown in Fig. 8A, MT-2Org cells, CB1 cells that had been subjected to continuous exposure to CH fibers and then removed from contact with CH fibers for one week prior to this assay, and KD4 knockdown and Scr clones were exposed to 0, 50, 100 and 200 μg/ml CH fibers. Results revealed that MT-2Org and KD 5 clone tended to display a dose-dependent increase in ROS production, unlike the case with CB1 and Scr.

Additionally, the NNT inhibitor palmitoyl CoA-K was added to CB1 and CR1 cultures (5 × 10^5 cells) and cultured with inhibitor for 4 h in the presence of 100 μg/ml CH or CR fibers, respectively. Cultures with control reagent (Kcl) were then compared. As shown in Fig. 8B, the presence of inhibitor induced a decrease in ROS production in continuously exposed sublines. Although we have not confirmed that palmitoyl CoA-K crosses the plasma membrane in our experiments, we applied the past literature (Lopert and Patel, 2014). In addition, unfortunately we did not measure NNT activity in palmitoyl CoA-K treated cells. Taken together, NNT acts to reduce the level of ROS following asbestos exposure.

3.8. Changes in NADPH/NADP+ and GSSG/GSH ratios following silencing of NNT

Finally, intramitochondrial NADPH/NADP+ and GSSG/GSH ratios were compared among MT-2Org, CB1 and the NNT knockdown clone. MT-2Org, CB1 and KD5 cells (2 × 10^7) were cultured in 6 cm dishes for 18 h in the absence or presence of 50 μg/ml CH fibers. As shown Fig. 9A, the NADPH/NADP+ ratio showed higher tendency in CB1 compared to MT-2Org cells, although statistical significance was not found. Silencing of NNT resulted in recovery of this ratio to that observed in MT-2Org cells. This indicated that overexpression of NNT accelerated NADP+ to NADPH conversion and reduced the level of ROS in mitochondria. Re-exposure of cells to CH fibers did not affect this ratio.

However, as shown in Fig. 9B, the GSSG/GSH ratio did not change significantly in cells with or without re-exposure to CH fibers. Taken together, it was determined that overexpression of NNT in a continuously exposed subline of MT-2Org increased NADPH to NADP+ conversion and reduced the level of ROS in mitochondria. The lack of marked alteration in the GSSG/GSH ratio may result from continuous exposure to asbestos fibers and sustained production of ROS in exposed cells, notwithstanding the high performance of NNT in mitochondria. It seemed that enhancement of NNT activity may have prevented the cytotoxic effects of ROS that were generated following continuous exposure to asbestos fibers.
4. Discussion

The immunological effects of asbestos fibers have yet been fully investigated. The chemical core elements of asbestos fibers comprise Si and O2 (Kohyama et al., 1996; Sporn, 2011), and patients who have silicosis as a result of exposure to silica can have complications typically observed with various autoimmune diseases such as rheumatoid arthritis, systemic sclerosis and anti-neutrophil cytoplasmic antibody (ANCA) related vasculitis/nephritis (Pollard, 2016; Steenland and Goldsmith, 1995; Lee et al., 2017b). Thus, asbestos fibers may affect human immune cells, although the physical characteristics of silica may differ (particulate versus fibrous).

Further questions were then raised. Given that asbestos such as iron-containing CR fibers in addition to CH fibers can induce ROS production in asbestos-exposed cells, the expression of antioxidative stress-related molecules in MT-2Org and sublines such as CB1 and CR1 was investigated. Additionally, the OXPHOS system may play a role in exposed cells in rendering harmless the ROS produced (Rydström 2006; Papa et al., 2012; Ciccarese and Ciminale, 2017; Maio et al., 2017; Salazar, 2018). Hence, differences in the expression of OXPHOS system components requires investigation.

Regarding the expression of antioxidative stress-related molecules, only thioredoxin levels seemed to be lower in continuously exposed sublines compared to MT-2Org cells untreated with asbestos fibers. Thioredoxins are known to act as antioxidants (Powis and Montfort, 2001a, 2001b; Chong et al., 2014). These proteins are involved in cysteine thiol-disulfide exchanges. A recent report indicated that thioredoxin-1 improves the immune-metabolic phenotype of anti-tumor immunity from the viewpoint of the surrounding oxidative tumor microenvironment (Chakraborty et al., 2019). The report showed that overexpression of thioredoxin in T cells prevented microenvironment-induced oxygen stress and thioredoxin-overexpressing T cells showed reduced glucose uptake and decreased effector function (Powis and Montfort, 2001b). Additionally, clustering of tumor reactive T cells with high concentrations of thioredoxin in an animal model demonstrated improved tumor control (Powis and Montfort, 2001b). Regarding thioredoxin and anti-tumor immunity, decreased expression of thioredoxin may be correlated with the reduction in anti-tumor immunity found in sublines continuously exposed to asbestos (Kumagai-Takei, et al.; 2011, 2018; Matsuzaki et al., 2012). However, the reason why only thioredoxin was down-regulated in asbestos-exposed sublines of all the molecules examined related to antioxidative stress remains unclear. Further investigations are required in an effort to delineate the correlation between thioredoxin and anti-tumor immunity found in these sublines.

Regarding the OXPHOS system and NNT, the most remarkable change was upregulation of NNT in sublines. Thus, the manner by which upregulated NNT contributed to the cellular and molecular characteristics in sublines continuously exposed to asbestos required further investigation. Panel B shows ROS production in CB1 or CR1 sublines following exposure to NNT-inhibitor palmitoyl CoA-K or KCl (control). Asterisks indicate statistical significance $p < 0.05$ by student’s T test.
cells, which we would like to address in the future. Because the high expression of NNT in asbestos-exposed long-term low-strain strains was the most important finding in this study, the mechanism for acquiring resistance to asbestos-induced apoptosis in sublines is probably different. It seems that several phenotypes following a reduction in anti-tumor immunity could potentially be involved in subsequent cellular events to elicit cell survival following recurring encounters with asbestos fibers.

ROS production in NNT knockdown clones showed similar patterns to MT-2Org in a dose-dependent manner following exposure to various concentrations of CH fibers. Furthermore, addition of the NNT inhibitor palmitoyl CoA-K to sublines resulted in increased ROS production. These findings indicated that continuously exposed sublines prevent cellular damage usually induced by ROS production following exposure to asbestos fibers by upregulating NNT. Although CB1 subline showed an increased tendency in NADPH/NADP+ ratio, the same ratio was reduced in the NNT knockdown clone as in MT-2Org. However, the GSSG/GSH ratio, which is driven by NADPH to NAPD+ conversion, remained unchanged in MT-2Org, CB1 and the NNT knockdown clone.

The GSH/GSSG cycle contributes towards rendering harmless the ROS produced by continuous exposure to asbestos fibers. It would be better to measure the peroxiredoxin 3 dimer/monomer ratio as a secondary readout of 'oxidative stress' in the future for the detailed understanding regarding alteration in T cells exposed to low dose of asbestos fibers continuously. Thus, sublines upregulated NNT to prevent cellular damage that could be caused by continuously produced ROS. Sublines were subjected to continuous ROS production by exposure to asbestos. Resistance to asbestos-induced apoptosis was based on a balance between upregulation of NNT and production of ROS caused by asbestos.

Given that T cells continuously exposed to asbestos fibers showed a reduction in anti-tumor immunity, it would be better to prevent these T cells from surviving when encountering chronic, recurrent and repeated exposure to asbestos fibers in the lung and lymph nodes where inhaled and undigested fibers can remain for a long time. Findings pertaining to exposure of T cells to low-dose fibers seemed to reflect our experimental cell line model, which included a reduction in CXCR3 surface expression and decreased potential of IFN-γ production, and confirmed the peripheral blood T cells derived from asbestos-exposed patients with PP

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**Fig. 9.** Panel A shows changes in NADP+ (white box) and NADPH (gray box) in MT-2Org, CB1 and KDS cells exposed 0 or 50 μg/ml CH fibers for 18 h. The table on the right shows changes in the NADPH/NADP+ ratio within these cells. No statistical significance (n.s.) was found, although CB1 seemed to be higher ratio compared with MT-2Org and KDS. Panel B shows the GSSG (Black box)/GSH (white box) ratio under the same conditions for MT-2Org, CB1 and KDS cells. The percentage of GSSG was very low in all cells, untreated and treated with CH fibers, the right panel shows zoomed GSSG percentages. However, as shown in the lower table, no statistical significance was found.

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| NADPH/NADP+ Ratio |
|--------------------|
| Cell Line | CH Exp. | Ave | SD |
| Org | 0 | 0.459 | 0.131 |
| CB1 | 50 | 0.438 | 0.146 |
| KDS | 0 | 1.072 | 0.293 |
| KDS | 50 | 1.149 | 0.176 |
| n.s. |

| GSSG/GSH Ratio |
|----------------|
| Cell Line | CH Exp. | Ave | SD |
| Org | 0 | 0.0123 | 0.0112 |
| CB1 | 50 | 0.0189 | 0.0153 |
| KDS | 0 | 0.0129 | 0.0143 |
| KDS | 50 | 0.0276 | 0.0375 |
| n.s. |
or MM. One approach might be to inhibit NNT in T cells of asbestos-exposed patients, since our experimental findings revealed knockdown of unregulated NNT did not affect proliferation or the occurrence of apoptosis. If NNT were to be inhibited in asbestos-exposed patients, these T cells may survive in a manner similar to T cells that had not encountered fibers. If these cells are then activated by some antigens, they may die by activation-induced cell death. Additionally, cell death could be induced by ROS production in T cells or the surrounding microenvironment, and NNT-inhibited T cells may die as usual. However, T cells chronically exposed to asbestos may survive longer because of resistance to asbestos/ROS-induced cell death and modified cellular features that reduce anti-tumor immunity. Although this scenario is consistent with the findings presented in this study, there is a plethora of areas that have yet to be investigated. This recent report showed that NNT deficiency impeded proliferation of cancer cells. However, care is required when considering approaches that rely on permanent NNT deficiency in immune cells by genome editing or other means since this may also cause some type of immune-deficient state in humans. A better approach might be to search for substances in foods or identify physiologically active materials that reduce NNT, so that if people become exposed to asbestos, these chemo-preventive methods to reduce NNT in T cells may assist in the recovery or maintenance of anti-tumor immunity to prevent the occurrence of asbestos-induced cancers.

Further studies are required in an effort to identify these substances and investigate the effects on NNT expression and function in T cells continuously exposed to asbestos. This should lead to the development of effective strategies to inhibit the onset and progression of asbestos-induced cancers by augmenting anti-tumor immunity.

5. Conclusion

These results indicated that NNT is a key factor in preventing ROS-induced cytotoxicity in T cells continuously exposed to asbestos. Considering that these sublines showed a reduction in anti-tumor immunity, modification of NNT may contribute to recovery of the anti-tumor effects in asbestos-exposed T cells.

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Declaration of Competing Interest

All authors have no disclosure of COI for this study.

Ethical approval and consent to participate

Since all of experiments in this study were used only cell line and its sublines, there was no necessity to obtain ethical approval.

Consent for publication

All authors agreed with consent for publication.

Availability of supporting data

We do not include any supporting data.

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