Asbestos conceives Fe(II)-dependent mutagenic stromal milieu through ceaseless macrophage ferroptosis and β-catenin induction in mesothelium

Fumiya Ito a, Izumi Yanatori a, Yuki Maeda a, Kenta Nimura a, Satoki Ito a, Tasuku Hirayama b, Hideko Nagasawa b, Norihiko Kohyama c, d, Yasumasa Okazaki a, Shinya Akatsuka a, Shinya Toyokuni a, e, f, * a Department of Pathology and Biological Responses, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550, Japan b Laboratory of Pharmaceutical and Medicinal Chemistry, Gifu Pharmaceutical University, Gifu, 501-1196, Japan c Faculty of Economics, Toyo University Graduate School of Economics, Tokyo, 112-0001, Japan d National Institute of Occupational Safety and Health, Kawasaki, 214-8585, Japan e Center for Low-temperature Plasma Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8603, Japan f Sydney Medical School, The University of Sydney, NSW, 2006, Australia

ARTICLE INFO

Keywords:
Asbestos
Iron
Ferroptosis
Lysosomal cell death
p16INK4A

ABSTRACT

Asbestos is still a social burden worldwide as a carcinogen causing malignant mesothelioma. Whereas recent studies suggest that local iron reduction is a preventive strategy against carcinogenesis, little is known regarding the cellular and molecular mechanisms surrounding excess iron. Here by differentially using high-risk and low-risk asbestos fibers (crocidolite and anthophyllite, respectively), we identified asbestos-induced mutagenic milieu for mesothelial cells. Rat and cell experiments revealed that phagocytosis of asbestos by macrophages results in their distinctive necrotic death; initially lysosome-dependent cell death and later ferroptosis, which increase intra- and extra-cellular catalytic Fe(II). DNA damage in mesothelial cells, as assessed by 8-hydroxy-2-deoxyguanosine and γ-H2AX, increased after crocidolite exposure during regeneration accompanied by β-catenin activation. Conversely, β-catenin overexpression in mesothelial cells induced higher intracellular catalytic Fe(II) with increased G2/M cell-cycle fraction, when p16INK4A genomic loci localized more peripherally in the nucleus. Mesothelial cells after challenge of H2O2 under β-catenin overexpression presented low p16INK4A expression with a high incidence of deletion in p16INK4A locus. Thus, crocidolite generated catalytic Fe(II)-rich mutagenic environment for mesothelial cells by necrotizing macrophages with lysosomal cell death and ferroptosis. These results suggest novel molecular strategies to prevent mesothelial carcinogenesis after asbestos exposure.

1. Introduction

Asbestos, a natural fibrous nanomaterial [1], has been abundantly used worldwide for various industrial purposes, but induces distinct respiratory pathologies, including pulmonary fibrosis, lung cancer and malignant mesothelioma (MM) decades after exposure. Thus, asbestos is still a huge social burden worldwide [2–4]. Asbestos-associated carcinogenesis has been studied from two distinct standpoint, direct and/or indirect effects [5]. Direct effects suggest that asbestos fibers are phagocytosed by mesothelial cells, reach inside the nucleus and generate mutations almost physically [6–8]. On the other hand, indirect effects hypothesize that asbestos fibers localize inside macrophages and that oxidative stress from frustrated phagocytosis induces mesothelial genetic alterations [9] through activation of Nalp3 inflammasome [10] and tissue remodeling processes [11].

Pathogenicity of asbestos fibers has been associated with iron [4, 12–14], as exemplified by asbestos body in histology [15]. Intraperitoneal injection of ferric saccharate induces MM in wild-type rats [16,17], and more recently continued oxidative stress via Fenton reaction [18] in the kidney of wild-type rats leads to early p16INK4A deletion [19], resulting in renal cell carcinoma [20,21]. These reports suggest that excess iron plays a role in carcinogenesis [22,23]. Macrophages play a crucial role in iron regulation, including iron recovery in spleen from senescent red blood cells. Thus, macrophage dysfunction may contribute to carcinogenesis through iron-dependent oxidative stress. However,
little is known concerning the molecular mechanisms of asbestos-induced mesothelial carcinogenesis surrounding excess iron. Regarding the mutation spectrum collected using human samples, ~70% of MM exhibits p16\(^{INK4A}\) homozygous deletion [4,24–28], which is nowadays used for the pathological diagnosis of MM [29,30]. The rat model also provides the same deletion [31]. Here we for the first time demonstrate that ceaseless macrophage necrotic death, through lysosomal cell death and ferroptosis [32,33], generates catalytic Fe(II)-rich stromal mutagenic microenvironment for mesothelial cells and further that the associated activation of β-catenin is advantageous in mesothelial cells to obtain deletion of p16\(^{INK4A}\) via increased fraction of G2/M phase, increased intracellular catalytic Fe(II) and juxta-nuclear membrane position of p16\(^{INK4A}\) genomic loci.

2. Materials and methods

2.1. Animal experiments

Asbestos-induced peritonitis model was produced as previously described [31]. Briefly, male Fischer-344 rats (SLC Japan, Shizuoka, Japan; 6 wks old; 140–150 g body weight) were injected intraperitoneally (ip) with a suspension of 10 mg asbestos (crocidolite [UICC], anthophyllite [34]). We collected peritoneal lavage (PL; 30 ml physiological saline, containing 200 μM Tris amine 2-pyridyl methyl [Sigma-Aldrich] for stabilization of Fe(II)). Mesothelial cells were collected from rat mesentery as previously described [35]. We dissected organs for histological analysis at autopsy after euthanasia. The animal experiment committee of Nagoya University Graduate School of Medicine approved the experiments.

2.2. Cell culture

Met5A human mesothelial cell line (ATCC), LP9 human mesothelial cell line (Coriell Institute), Met5A-derived stable cell lines expressing Fucci [36], mouse RAW264 and human THP1 macrophage cell lines and HEK293T cell line (RIKEN Cell Bank, Tsukuba, Japan) were grown under standard sterile cell culture conditions (37 °C, humidified atmosphere, 5% CO\(_2\) in RPMI1640 [Wako, Osaka, Japan], containing 10% fetal bovine serum [FBS; Biowest, #S1780; Nuaill, France] and 1% antibiotic-antimycotic (Gibco). Cells were used within two months after thawing. In the co-culture experiments, THP-1 or RAW264 cells were differentiated in 6 transwell inserts (membrane pore size of 0.4 μm, Corning, #3450). Macrophages and Met5A cells were co-cultured in CO\(_2\) incubator with RPMI1640 medium with 1% FBS for 48 h prior to incubation with or without asbestos.

2.3. Microarray-based gene expression analysis

Microarray analysis was performed, using Agilent SuperPrint G3 Rat GE Ver 2.0 8x60 K Microarray slides (G4858A#74036, Agilent Technologies, Santa Clara, CA) and Low Input Quick Amp Labeling kit, using Agilent’s one-color Microarray-based Gene Expression Analysis protocol (5190-2305, Agilent Technologies). Total RNA was extracted from rat mesothelium 1 wk after crocidolite injection. Amplified cRNA was labeled with Cy3-CTP. Hybridization was done in a hybridization oven at 65 °C for 17 h with rotation. Microarray slides were then washed with GE wash buffer and scanned. Data were analyzed with GeneSpring FX 13.1 software (Agilent Technologies).

2.4. GSEA (gene set enrichment analysis)

Based on the entire microarray profiles using predefined gene sets, GSEA exploits novel computational methods [37,38]. Pre-defined gene set in the Molecular Signatures Database (MsigDB) was selected for analyses; the C5 set, a Gene Ontology (GO) molecular function gene set derived from the Molecular Function Ontology database in MSigDB was used. The gene sets included in the analysis were limited to those that contained between 10 and 500 genes. Permutation was conducted 1000 times according to default-weighted enrichment statistics and by using a signal-to-noise metric to rank genes according to their differential expression levels across the no-treatment and crocidolite (1 wk) groups. Significant gene sets were defined as those with a nominal P-value <0.05.

2.5. Cloning

Cloning was performed using chemically competent E. coli, derived from Stbl3 Competent Cells (C73793, Thermo Fischer). Correct cloning and integration into target vectors were confirmed with sequencing.

2.6. Cloning of components for endogenous β-catenin

CMV promoter of pcDNA3-EGFP (Addgene) was replaced by Cag promoter (PX461, #48140, addgene) to enhance promoter activity in mesothelial cells. Cag-pcDNA3-EGFP construct was generated, using a general protocol with restriction enzymes. Insert products were

---

**Abbreviations**

ANOVA analysis of variance  
CAS CRISPR-associated proteins  
CTSB cathepsin B  
DAPI 4',6-diamidino-2-phenylindole  
DFO deferoxamine  
DIM differential interference contrast microscope  
DMT1 divalent metal transporter 1  
DSB double-strand break  
FAC ferric ammonium citrate  
FACS fluorescent activated cell sorter  
FBS fetal bovine serum  
FISH fluorescent in situ hybridization  
FITC fluorescein isothiocyanate  
FL full length  
FSC forward scatter  
GFP green fluorescent protein  
GTX glutathine peroxidase  
GSEA Gene Set Enrichment Analysis  
HNE 4-hydroxy-2-nonenal  
LDCD lysosome-dependent cell death  
LDH lactate dehydrogenase  
LMP lysosomal membrane permealization  
MHC major histocompatibility complex  
MM malignant mesothelioma  
NOX NADPH oxidase  
OHDG 8-hydroxy-2-deoxyguanosine  
PEI polyethyleneimine  
PL peritoneal lavage  
PRE post-transcriptional regulatory element  
ROS reactive oxygen species  
SSC saline sodium citrate  
SSC side scatter  
TF transferrin  
TFR1 transferrin receptor 1  

obtained by PCR amplification. After ligation of these components, the product was used for transformation, from which plasmids were recovered and verified by sequencing.

2.7. shRNA knockdown of β-catenin

shRNA was used to knock down β-catenin expression in Met5A cells. β-catenin shRNA vector (pLKO.1 shβ-catenin) was constructed according to the protocol of pLKO.1 puro vector (#8453, Addgene). pLKO.1 shβ-catenin or scramble [Supplementary Table 3], packaging plasmid pCMV-VSV-G-RSV-Rev (RD804393, RIKEN BRC) and pCAG-HIV-gp (RD804394, RIKEN BRC) were co-transfected into the 293T cells, by using the polyethylenimine (PEI; 23966-1, Polyscience, Inc.) according to the manufacturer’s protocol and culture medium was replaced 3 h after transfection. Met5A cells were seeded in 12-well plates and transduced in RPMI1640, containing 10% FBS. The cells were reseeded in 6-well plates 12 h after incubation and selected using 250 ng/ml puromycin for 7 days. Knockdown efficiency was confirmed with western blotting.

2.8. Transient overexpression of β-catenin

1 × 10⁶ Met5A cells were seeded in a 6-well plate 1 day before transfection. 1 μg of the plasmid construct of full length-β-catenin and Δβ-catenin were diluted in 300 μl OptiMEM (Thermo Fischer) and prepared for transfection with 3 μg of PEI with 10 min incubation. The same transfection mix was prepared with an empty vector as a control (mock, pcDNA3-EGFP; #13031, Addgene). The transfection mixtures were added to the cells at a confluence of 80–90%. On the next day, the medium was exchanged to fresh medium. After 48 h incubation, these cells were used for imaging, FACS, and western blotting analyses.

2.9. LDH cytotoxicity assays

Cytotoxicity was evaluated with a LDH-cytotoxicity detection kit (Roch Diagnostics). Met5A and THP1 cells were seeded in a 96-well tissue culture plate at a density of 1.5 × 10⁴ cells per well and incubated with medium containing 10% FBS. Prior to crocidolite exposure, we exchanged the medium to 1% FBS with or without iron (ferric ammonium citrate, FAC; Sigma) at different concentrations per well. After 48–72 h, the supernatant was used for LDH assay (cytotoxicity), and cells were used for WST assay (cell viability). The absorbance of the samples was calculated at each wavelength with a plate reader (PowerScan4 or Multiscan FC). Cytotoxicity was calculated using the formula in the kit protocol.

2.10. Cell proliferation assay

A proliferation kit (WST; Cell Count Reagent SF, Nacalai Tesque, Kyoto, Japan) was used to check cell viability. The assay used highly water-soluble tetrazolium salt. Met5A cells were seeded in a 96-well plate at a density of 1.0 × 10⁴ cells per well and incubated with RPMI1640 medium with 1% FBS. After 48 h, 10 μl of crocidolite suspension at different concentration per well were added. After 24, 48 or 72 h, 10 μl of Cell Count Reagent SF was added to medium and incubated for 2.5 h at 37 ºC under protection from light. Absorbance at 450 nm was measured with a microplate reader.

2.11. In vitro stimulation of macrophage

For stimulation of macrophage in vitro, RAW 264 or THP1 cells were plated at 1 × 10⁴ cells/dishes in 6-well plate in the presence of phorbol 12-myristate 13-acetate (PMA, 10 μM). After 12 h, cells were stimulated either with IFN-γ (100 ng/ml, PeproTech), IL-4 (20 ng/ml, PeproTech), LPS (10 ng/ml, Sigma), IgG (Jackson ImmunoResearch) coated wells + LPS (100 ng/ml) or TGF-β (0.5 ng/ml, R&D Systems) as previously described [39,40]. After stimulation, these cells were used for q-PCR, western blotting, cell death assay and transmission electron microscopy.

2.12. Flow cytometry (FACS)

Inflammatory cells were collected from rat peritoneal lavage (PL) as described [41]. The harvested cells were rinsed with centrifugation (200 × g, 3 min); the supernatant was used for detection of extracellular catalytic Fe(II) with plate reader whereas the pellet was used for FACS analysis. SiRhoxNox-1 solution (final concentration 2 μM) [42–44] and Hoechst 33342 (Thermo Fischer) were added to the tubes containing cells, followed by incubation in the dark at 37 ºC. The cell analysis and sorting were carried out, using FACS Aria II (BD Biosciences). Hoechst 33342-negative cells were removed as dead cells. As reported previously [41], we identified macrophage-subset with forward and side scatters, followed by an analysis on catalytic Fe(II). Fluorescent signals were measured at 370 nm (450/50) for Hoechst33342 and at 633 nm (660/40) for SiRhoxNox-1. All flow cytometric data were analyzed with FACS Diva version 8.0.1.

2.13. Transmission electron microscopy

Stimulated THP1 cells as described were plated at 1 × 10⁶ cell/dish in 35-mm tissue culture dishes. These cells were exposed to asbestos (15 μg/cm²) by incubating for 24–48 h. Cells were fixed with 2.5% glutaraldehyde (TAAB) in 0.1 M Sorenson’s buffer (0.1 M H₂PO₄, 0.1 M HPO₄ [pH 7.2]) for at least 1.5 h, and then treated with 1% OsO₄ in 0.1 M Sorenson’s buffer. After dehydration through an ethanol series, cells were embedded in Epon-812 (TAAB). A thin section was cut on an MT-7000 ultra-microtome, stained with 1% uranyl acetate and 0.4% lead citrate and then examined under a JEOL JEM-1400 EXII electron microscope (JEOL, Tokyo, Japan). Pictures were taken on an ORCA-HR digital camera (Hamamatsu Photonics, Hamamatsu, Japan) at 5000–50,000-fold magnification and measurements were performed, using the AMT Image Capture Engine.

2.14. Western blot analysis

Cells were lysed with a lysis buffer (1 M NaCl, 50 mM Tris-HCl, 0.1% SDS, pH 8.0, 0.5% sodium deoxycholate and 1% NP-40) supplemented with a protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Protein extraction, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or Tricine SDS-PAGE were performed as previously described [45]. The antibodies used are described in the Supplementary Table 1.

2.15. Quantitative real-time PCR

Total RNA was isolated from cultured cells using RNeasy plus kit (Qiagen). cDNA was synthesized from 4 μg of RNA using SuperScript III cDNA synthesis kit (Thermo Scientific). qPCR was performed in triplicates with 1 μl diluted cDNA (1:5) using SYBR green Fast mix (Thermo Scientific) on an iCycler RT-PCR detection system (BioRad). Expression was normalized to Actb or Gapdh. Expression levels were measured, using primers purchased from Thermo Fischer Scientific, and all the primer sequences are described in the Supplementary Table 2.

2.16. FISH analysis

For detecting p16⁰⁰⁰⁰⁰ deletion in the rat mesothelial cells, bacterial artificial chromosome clone RNBl-039C01 (p16⁰⁰⁰⁰⁰ as a test probe) and RNBl-266D03 (rat chromosome 5 centromeric region as a reference probe) were extracted and used for FISH analysis by labeling with biotin-16dUTP (TAKARA) or digoxigenin-11-dUTP (Enzo life Science) in general nick translation methods. The size of the labeled probe was confirmed by agarose gel electrophoresis to be 200 to 400 bp. After
ethanol precipitation (20 μl of products, 50 μl of 4 M ammonium acetate, 2.0 μl of 25 μg/μl salmon sperm DNA [Wako], 10 μl of 4 μg/μl of E.coli rRNA and 500 μl of ethanol) at −80 °C for 30 min, the labeled probe was dissolved in formamide. Rat Cot-I DNA (Chromosome Science Lab) was added (20 μg/μl) to the hybridization mixture to minimize background signal. Stock slides were completely air-dried, followed by pepsin treatment (10 μg/ml in 10 mM HCl, pH 2.0) at 37 °C. Hybridization was performed at 37 °C overnight, followed by washing with 2 × SSC at 42 °C at room temperature, sequentially. Finally, avidin-FITC (Vector; 1:100 dilution) or anti-digoxigenin antibody (Vector; Dylight594-labeled anti-Digoxigenin/Digoxin, 1:200 dilution) and DAPI was applied for nuclear counterstaining.

2.17. Fucci stably expressing mesothelial cells (fMet5A)

The genes encoding orange or green Fucci indicators were amplified with PCR from the pFucci-G1 orange vector or pFucci-S/G2/M green vector (MBL Life Sciences) and cloned into FUGW modified lentiviral vector (#14883, Addgene) with EcoRI and XbaI. Met5A cells were transduced by lentivirus encoding orange Fucci (G1-phase) and green
Fucci (S/G2/M-phase) and stable cell lines were cloned by limiting dilution method.

2.18. Cas FISH

Cas9 plasmid construct (pHAGE-TO-nls-st1dCas9-3nls-3XTagBFP2, #64512, Addgene) and sgRNA Expression vector (pLH-stgRNA3.1; #64118, Addgene) were subcloned as previously described [46]. For imaging, cells were grown on 35 mm glass-bottom dishes (3911-035; Iwaki, Shizuoka, Japan). Then, 150 ng of dCas9 plasmid and 750 ng of sgRNA plasmid were cotransfected using PEI, after which the cells were incubated for another 48 h. Fluorescent signals were measured at 488 nm (510/20) for Fucci-S/G2/M and 540 nm (570/20) and 370 nm (450/50) for BFPCas9 with a confocal microscope (LSM880, Carl Zeiss; Oberkochen, Germany). sgRNA sequences are described in Supplementary Table 4.

2.19. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) and an unpaired t-test with GraphPad Prism 5 software. Differences were considered significant when p < 0.05. The data are expressed as the means ± SEM (N = 3–10) unless otherwise specified. All the experiments were repeated at least three times.

3. Results

3.1. Only high-risk asbestos (crocidolite) induces iron-rich stromal milieu

Previously we showed in rats that crocidolite (blue asbestos) is highly carcinogenic causing MM [31] whereas a minor asbestos, anthophyllite, did not induce MM in our experiments (low-risk) [34]. To elucidate the cellular mechanism toward iron-rich stromal microenvironment, we first performed histological analysis in rats injected intraperitoneally either with high- or low-risk asbestos fibers. Four weeks after the asbestos injection, chronic granulomatous peritonitis as a reaction to foreign material was present in both the cases with mesothelial cells on granuloma and fibrosis, instead of attaching on the basement membrane. Polarizer analysis revealed that asbestos fibers were inside CD68-positive macrophages and not in mesothelial cells (Fig. 1a; Supplementary Fig. 1a and b). Of note, we observed necrotic death of macrophages only in the crocidolite group (Fig. 1b and c). 4-Hydroxy-2-nonenal (HNE) is a major product of lipid peroxidation [47, 48] and is one of the most sensitive markers for Fenton reaction [47, 49]. We detected HNE-modified proteins in both the granuloma, and quantified immunopositivity was significantly higher in the crocidolite granuloma than that of anthophyllite (Fig. 1d and e). Thus, we speculated that macrophages engulfing asbestos are responsible for chronic inflammation with generation of reactive oxygen species (ROS). In comparison to macrophages in peritoneal fluid (Supplementary Fig. 1a and b), macrophages in granuloma highly expressed M2-specific markers, such as CD163 and MHC II [50]. Both of them, whether crocidolite or anthophyllite, expressed NOX2, a major ROS producer in macrophages [51] (Supplementary Fig. 1a-c). However, intra-granuloma macrophages significantly accumulated iron only after crocidolite exposure (Fig. 1f and g). Peritoneal macrophages, iNOS-positive and M1 subtype (Supplementary Fig. 1a), collected iron most among all the inflammatory cells, and catalytic Fe(II) ([4] was significantly increased intracellularly and extracellularly only after crocidolite exposure (Fig. 1h, i, j; Supplementary Fig. 2). These data suggest that stromal iron overload is a feature of high-risk asbestos (crocidolite), resulting from macrophage necrosis (Supplementary Fig. 1c).

3.2. Crocidolite induces lysosome-dependent cell death in the initial phase and ferroptosis in the end phase in macrophages

To classify crocidolite-induced necrosis, we selected a macrophage cell line (THP-1 stimulated by PMA) for further analysis. We found that additional iron treatment decreased the cell viability of macrophages after asbestos exposure and enhanced necrotic death (Fig. 2a and b). We then performed a transmission electron microscopy to investigate which type of necrosis is induced by crocidolite. Crocidolite reached phagolysosome 24 h after exposure and permeabilized the phagolysosomal membrane whereas anthophyllite did not permeabilize it (Fig. 2c). Furthermore, we observed mitochondrial shrinkage after crocidolite exposure (Fig. 2d and e). Cathepsin B, a lysosomal enzyme, if present in nucleus or cytosol, causes lysosome-dependent cell death (LDCD) [33, 52]. We found cathepsin B localized to nucleus and cytosol of the macrophages engulfing crocidolite, which was enhanced with additional iron (Fig. 2f and g). Here, time-lapse analysis revealed that ROS surge in phagolysosome was followed by cytosolic increase in ROS and lysosomal increase in catalytic Fe(II) within a time-course of 120 min (Supplementary Fig. 4a-c). These results suggest that ROS originates not only from frustrated phagocytosis [11] but also from LDCD of macrophages.

From these observations, we speculated the involvement of ferroptosis [32] in crocidolite-induced macrophage cell death. Recent studies imply that cell death regulated by certain signal pathways in the initial phase may result in multiple forms of necrosis, ending in the rupture of plasma membrane [53]. To further elucidate the type of regulated necrosis, we performed protein analyses. Whereas ferroptosis- and necroptosis-associated proteins presented no significant changes in the initial phase of macrophage death by crocidolite, protein levels of Lamp2 and Cathepsin B increased time-dependently (Fig. 2h). Thus, crocidolite induced LCDC in macrophages at the initial-phase. At the end-phase 96 h after crocidolite exposure, Lamp2 and Cathepsin B increased (LDCD); GPX4 and TR1 decreased and ferritin increased (ferroptosis); RIP1 and MLKL increased (necroptosis). Whereas necroptosis inhibitor (necrostatin-1) and ferroptosis inhibitor (ferrostatin-1 and desferal [DFO]) partially suppressed the macrophage detachment and death, caspase inhibitor z-VAD-FMK), cathepsin B inhibitor (z-FA-FMK) and protease inhibitor (pepsatin A) were not effective (Fig. 2i and j; Supplementary Fig. 3a). These data suggest that macrophage necrosis ended in ferroptosis at the end phase.

3.3. Wnt/β-catenin pathway is activated during mesothelial regeneration

We next examined the biological responses of mesothelial cells after crocidolite exposure. In a rat model of intraperitoneal injection, we found mesothelial cells continuously synthesizing DNA (c-Myc-, Ki-67-, BrdU-positive cells) on the peritoneal surface (Fig. 3a and b). This regenerative activity of mesothelial cells continued at least from 24 h to 2 weeks after crocidolite exposure, when regenerative mesothelial cells were on the granuloma capturing crocidolite (Fig. 3a and b). What kinds of signaling pathways are activated during this mesothelial regeneration is still poorly understood [54, 55]. To clarify this, we performed gene set enrichment analysis (GO Accession No. GSE149285), using rat mesothelial cells collected from mesentry after exposure to crocidolite (Supplementary Fig. 5a-c). We discovered the association of Wnt/β-catenin pathway with mesothelial regeneration (Fig. 3c and d). Expression of CTNNB1 (catenin β1) was significantly increased in mesothelial cells 1 week after crocidolite exposure (Fig. 3e), which was confirmed with immunohistochemistry and mRNA analyses (Fig. 3b, c). Other overexpressed genes included c-Met and Tgfβ1.

3.4. Hydrogen peroxide source from macrophages is not limited to NOX2

To evaluate the ROS source in macrophages, we used THP-1 cells after PMA stimulation and analyzed NOX2 expression, a major source of H2O2 in macrophages [56, 57]. Both the peritoneal and intra-granuloma
macrophages expressed NOX2, which was not significantly different after exposure either to crocidolite or anthophyllite (Supplementary Fig. 1d–f). However, the amounts of H₂O₂ in the extracellular fluid were significantly increased only after crocidolite exposure when the macrophages were stimulated either with IFN-γ or IL-4 (Supplementary Fig. 1). Macrophages in granuloma expressed CD163 and MHC II, categorized as IL-4-stimulated macrophages (Supplementary Fig. 1c). Production of H₂O₂ increased not only in IFN-γ group (M1 subtype) but also in IL-4 group (M2a subtype), whereas Nox2 expression was low (Supplementary Fig. 1d–f). These results suggest that the source of

Fig. 2. Crocidolite induces macrophage necrosis via lysosome-dependent cell death at an early phase and later via ferroptosis. (a, b) Cell viability (WST) and necrosis ratio (LDH) assays reveal higher toxicity of crocidolite than anthophyllite for THP1 macrophage in the presence of excess iron. Crocidolite or anthophyllite asbestos was exposed to THP1 macrophage cells in the presence of 10 μg/ml ferric ammonium citrate (FAC) for 48 h. (c) Transmission electron microscopy of M0-state THP1 macrophage exposed to asbestos for 24 h. Crocidolite causes lysosomal rupture whereas anthophyllite fibers are confined inside lysosome (bar = 5 μm). (d, e) Transmission electron microscopy of mitochondrial damage in THP1 after crocidolite exposure. Significantly shorter mitochondria are observed (bar = 200 nm). NT, no-treatment. (f, g) Nuclear localization of lysosomal cathepsin B (CTSB) in IL-4 stimulated THP1 cells after exposure to crocidolite (25 μg/cm²) and 100 μg/ml FAC. White arrowhead, nucleus; cathepsin B, red; nucleus, cyan (bar = 50 μm). (h) Time-course immunoblot analysis reveals the involvement of ferroptosis. IL-4 stimulated THP1 was exposed to crocidolite (25 μg/cm²). (i) Immunoblot analysis of IL-4 stimulated THP1 72 h after crocidolite exposure (25 μg/cm²) in the presence of various inhibitors. Necrostatin-1; (necroptosis inhibitor, 40 μM), Ferrostain-1; DFO, deferoxamine (ferroptosis inhibitors; 500 nM and 400 μM, respectively), zVAD-FMK (apoptosis inhibitor, 184 μM), z-FA-FMK (lysosome-dependent cell death inhibitor, 20 μg/ml), Pepstatin-A (autophagic cell death inhibitor, 20 μg/ml). (j) LDH assay in IL-4 stimulated THP1 exposed to crocidolite in the same condition as in (i) (means ± SEM; N ≥ 6). Refer to text for details. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
extracellular H$_2$O$_2$ is not limited to NOX2.

3.5. Necrotic macrophages induce iron-dependent oxidative damage to distant mesothelial cells

Necrotizing macrophages produced extracellular H$_2$O$_2$ (Supplementary Fig. 1f). To determine whether crocidolite indirectly induces mesothelial cytotoxicity via injured macrophages, we performed in vitro experiments, using double chamber co-culture system between mesothelial cells and macrophages (Fig. 4a). H$_2$O$_2$ from macrophage necrosis indirectly caused lipid peroxidation in mesothelial cells (Fig. 4b-d). 8-Hydroxy-2'-deoxyguanosine (8-OHdG) is a marker of oxidative DNA damage [58-60]. We observed that nuclear 8-OHdG in mesothelial cells increased in the same system in an iron concentration-dependent manner (Fig. 4e and f). Furthermore, in rat experiments, nuclear 8-OHdG was observed in mesothelial cells sitting on granuloma and fibrosis only after crocidolite exposure (Fig. 4g and h).

3.6. Stabilization of β-catenin orchestrates iron metabolism through the cell cycle

To elucidate the association between β-catenin and iron metabolism in mesothelial cells especially from the viewpoint of the cell cycle, we used β-catenin overexpression system (Fig. 5a). β-Catenin overexpression in mesothelial cells significantly increased intracellular catalytic Fe(II) in a dose-dependent manner in comparison to the mock transfection group (Fig. 5b and c). Lithium Chloride (LiCl) inhibits GSK-3β which works for degradation of β-catenin through phosphorylation to Ser33, 37 and Thr41 [61]. We confirmed an increase in β-catenin expression by LiCl or hWnt with time-dependence. Simultaneously, we observed a gradual increase in expression of transferrin receptor 1 (TfR1) (Fig. 5d and e). Phosphorylation of β-catenin at Ser675 (pβ-catenin-S675) decreases β-catenin stabilization and nuclear translocation, starting transcriptional activity for proliferation. The cells with nuclear β-catenin accumulation revealed significantly increased catalytic Fe(II). We
categorized the immunostaining pattern of β-catenin into 4 different types (low expression, [plasma] membrane, nuclear and high expression) in increasing orders (Fig. 5 f and g). These results suggest the association of TfR1 expression with β-catenin-dependent Fe(II) uptake. Furthermore, we found that β-catenin expression and catalytic Fe(II) were linear in correlation with DNA amount per cell (Fig. 5 h and i). We also found that TfR1 expression and pβ-catenin-S675 are in linear correlation. Thus, overexpressed β-catenin (Δ89 as degradation recognition site-deficient β-catenin) contributes to and accommodate an iron-rich status in mesothelial cells with increased ferritin, an iron storage protein (Fig. 5 j) whereas β-catenin knockdown decreased iron import and storage (Fig. 5 i). In summary, β-catenin overexpression led to increased intracellular catalytic Fe(II), which was at the highest at G2/M phase.

3.7. β-Catenin-overexpressing mesothelial cells suffer iron-dependent DNA double-strand breaks

DNA double-strand breaks (DSBs) is a major event during the production process of carcinogenic mutations and deletions. DSBs recruit γH2AX to the DSB foci [60]. In in vitro analysis using Met5A mesothelial cells, γH2AX expression was induced in an iron-dependent manner in the presence of H$_2$O$_2$ (Fig. 6a). Immunohistochemical analysis revealed that γ-H2AX immunopositivity is significantly higher in cells with high β-catenin levels, which were often associated with mitosis (Fig. 6 b and c). Upon β-catenin stabilization with LiCl, γH2AX levels further increased in β-catenin-overexpressed mesothelial cells in the presence of iron and H$_2$O$_2$ (Fig. 6d). On the peritoneal surface of granuloma in rats, regenerative mesothelial cells strongly showed DSBs after crocidolite exposure whereas those after anthophyllite exposure did not (Fig. 6e).

3.8. Asbestos microenvironment induces p16$^{INK4A}$ deletion in repairing mesothelial cells

Nuclear localization of the genomic loci is critical for the deletion process because catalytic Fe(II)-dependent reactive species attacks DNA more from the nuclear periphery and less to the center [62]. We hypothesized that the p16$^{INK4A}$ deletion events occur in the early stage of mesothelial carcinogenesis. To detect nuclear localization of p16$^{INK4A}$ in...
Fig. 5. β-Catenin overexpression increases catalytic Fe(II) in mesothelial cell by modifying iron metabolism and cell cycle.

(a) Scheme of β-catenin overexpression system as a fusion protein with GFP. FL, full-length; Δ89-β-catenin, N-terminal 89-amino acids were deleted for stabilization of β-catenin. PRE, post-transcriptional regulatory element. (b, c) Catalytic Fe(II) (SiRhoNox-1 fluorescent probe, magenta) was significantly increased in β-catenin overexpressed Met5A mesothelial cell. (b, c) Immunoblot analysis of iron metabolism and β-catenin; LiCl (20 mM) and hWnt1 (20 ng/ml) for β-catenin activation. (d, g) Imaging for catalytic Fe(II) and β-catenin in FL-β-catenin (GFP) overexpressed Met5A cells. Expression pattern of β-catenin was classified into 4 types as follows from low to high; low expression, plasma membrane localization, nuclear localization and high expression. Catalytic Fe(II) levels were β-catenin dose-dependent in (g). (h, i) FACS analysis of β-catenin (GFP; green) and catalytic Fe(II) (SiRhoNox-1, magenta). Cell cycle was separated by DNA amount (Hoechst33342, blue) into G1, S and G2/M phases in FACS analysis. G2/M phase reveals high β-catenin expression and high catalytic Fe(II). (j) Immunoblotting analysis for the association of β-catenin levels with iron metabolism in Met5A cells. β-Catenin overexpression on the left increases intracellular iron and its knockdown on the right decreases it (NT, no treatment; Scr, scrambled; sh β-cat: β-catenin knock down; means ± SEM; N ≧ 4). Refer to text for details. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
system which can visualize a target gene using labeled-dCas9 (deficient each cell cycle, we used cell cycle indicator (Fucci) [36] and CasFISH (a) DNA double-strand breaks evaluated by Fig. 6. F. Ito et al.

We previously showed that anthophyllite, a minor iron-containing asbestos, presents a low risk for mesothelial carcinogenesis [34]. Based on the results, here we revealed for the first time, with differential animal experiments between high-risk and low-risk asbestos exposure (crocidolite and anthophyllite, respectively), that iron excess-induced mutagenic milieu is established for mesothelial carcinogenesis. Mesothelial cells cover somatic cavities as a layer of flat cells [15]. We discovered that the only mesothelial cells exposed to high-risk asbestos are sandwiched by this iron-rich milieu not only from the stromal side but also from the cavity side. The cavity side was full of catalytic Fe(II) and the stromal side showed massive macrophage necrosis as granuloma but also from the cavity side. The cavity side was full of catalytic Fe(II) and the stromal side showed massive macrophage necrosis as granuloma. We cannot suggest a response after iron depletion by DFO. In summary, stromal iron excess was derived from necrotized macrophages, at an early phase iron excess was derived from necrotized macrophages, at an early phase.

**4. Discussion**

We previously showed that anthophyllite, a minor iron-containing asbestos, presents a low risk for mesothelial carcinogenesis [34]. Based on the results, here we revealed for the first time, with differential animal experiments between high-risk and low-risk asbestos exposure (crocidolite and anthophyllite, respectively), that iron excess-induced mutagenic milieu is established for mesothelial carcinogenesis. Mesothelial cells cover somatic cavities as a layer of flat cells [15]. We discovered that the only mesothelial cells exposed to high-risk asbestos are sandwiched by this iron-rich milieu not only from the stromal side but also from the cavity side. The cavity side was full of catalytic Fe(II) and the stromal side showed massive macrophage necrosis as granuloma but also from the cavity side. The cavity side was full of catalytic Fe(II) and the stromal side showed massive macrophage necrosis as granuloma. We cannot suggest a response after iron depletion by DFO. In summary, stromal iron excess was derived from necrotized macrophages, at an early phase iron excess was derived from necrotized macrophages, at an early phase.
membrane protein (divalent metal transporter 1: DMT1) for intracellular iron [41], and iron should go through lysosomal/endosomal systems [42]. Additionally, lysosome is one of the most Fe(II)-rich cell compartments [68]. Thus, the lysosomal membrane permeabilization (LMP) is an important factor in iron turnover in senescent red blood cells, hepcidin regulation and lipid metabolism and Wnt/β-catenin signaling for mesothelial carcinogenesis. First, it is associated with oxidative damage with lipid peroxidation and an increase in 8-OHdG. A previous report suggested that the inflammatory and necrotic death and establishes catalytic Fe(II)-dependent stromal mutagenic milieu after exposure to crocidolite (left). Mesothelial regeneration after exposure to crocidolite accompanies β-catenin overexpression, which increases the risk of p16INK4A deletion via multiple mechanisms, such as increased G2/M phase fraction, higher intracellular catalytic Fe(II) and peripheral transfer of p16INK4A genomic loci in the nucleus (means ± SEM; N ≥ 4). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

completely rule out the possibility of minor overlap of other regulated cell death, such as apoptosis, pyroptosis or necroptosis [66,67].

Macrophage plays a central role in iron metabolism, including heme-iron turnover in senescent red blood cells, hepcidin regulation and lipocalin system [68]. Additionally, lysosome is one of the most Fe(II)-rich organella [41], and iron should go through lysosomal/endosomal membrane protein (divalent metal transporter 1: DMT1) for intracellular use [69]. Thus, the lysosomal membrane permeabilization (LMP) by crocidolite disturbs cellular iron metabolism, and macrophage cell death disseminates accumulated iron, eventually leading to iron excess in granuloma. It is critical that the macrophages cannot eliminate not only asbestos but also iron. Furthermore, the cells which collect necrotic debris are also macrophages. These vicious cycles induce continuous necrotic death and establishes catalytic Fe(II)-dependent stromal mutagenic environment.

Then, we focused on the mesothelial cells and screened with expression microarray for characteristic signalling during regeneration of crocidolite-induced mesothelial injury. β-Catenin was the most significant among the positive results, including c-Met and Tgfbr1. Double chamber culture system demonstrated a distant injurious effect of crocidolite-exposed macrophages to mesothelial cells, which revealed oxidative damage with lipid peroxidation and an increase in 8-OHdG.

Finally, we sought for possible links between β-catenin signal activation and p16INK4A deletion, a largest target gene of MM carcinogenesis [4,24–28]. We previously reported a positive autocrine loop between β-catenin and connective tissue growth factor in asbestos-induced rat MM [70,71]. There are three major merits in the activation of β-catenin signaling for mesothelial carcinogenesis. First, it is associated with regeneration. Thus, continued cell cycles with a higher fraction of G2/M phase increase mutation rate. Second, β-catenin modifies iron metabolism, increasing intracellular catalytic Fe(II) dose-dependently with β-catenin expression and especially at G2/M phase. This is consistent with a previous report, dictating the association between iron metabolism and Wnt/β-catenin pathway [72]. However, we for the first time analyzed the catalytic Fe(II) in association with cell cycle. Third, unexpectedly, genomic loci for p16INK4A are directed toward nuclear periphery at G2/M phase. Indeed, deletion of p16INK4A was demonstrated in mesothelial cells in the presence of high iron and H2O2 under high β-catenin expression. These results suggest that the inflammatory and...
mutagenic microenvironment by crocidolite selectively induced DNA DSBs in mesothelial progenitor or repairing cells, leading eventually to selective p16\(^\text{INK4A}\) deletion.

In conclusion, our findings highlight the importance of catalytic Fe (II)-dependent mutagenic stromal milieu in mesothelial genomic alterations that asbestos-induced indirect damage affects regenerative mesothelia with \(\beta\)-catenin-dependence. We discovered that mesothelial activation of \(\beta\)-catenin pathway during regeneration brings multiple advantages for their \(\beta\)-catenin-dependence. We discovered that mesothelial activation of \(\beta\)-catenin pathway during regeneration brings multiple advantages for their mesothelial carcinogenesis: toward the era of mesothelioma prevention, Free Inflammation 9 (2) (1985) 139–147.

Acknowledgments

This work was supported, in part, by JST CREST (Grant Number JPMJCR191H) and JSPS Kakenhi (Grant Number JP17H04064, JP19H03641, JP19H04623, and JP20H05502). Electron microscopic analyses were supported by Koji Itakura, Division of Medical Research Engineering, Nagoya University Graduate School of Medicine. We thank Nobuaki Misawa (Department Pathology and Biological Responses, Nagoya University Graduate School of Medicine) for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101616.

References

[1] A.F. Guaitieri, G.B. Andreozzi, M. Tomatis, F. Turci, Iron from a geochemical viewpoint. Understanding toxicity/pathogenicity mechanisms in iron-bearing minerals with a special attention to mineral fibers, Fre Radic. Biol. Med. 133 (2019) 21–37.

[2] WHO, IARC, Asbestos (chrysotile, amosite, crocidolite, tremolite, actinolite, and anthophyllite), in: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Lyon, France, 2012, pp. 219–371.

[3] S. Toyokuni, Role of iron in carcinogenesis: cancer as a ferrotoxic disease, Cancer. Res. 75 (2) (2015) 264–269.

[4] R. Bueno, E.W. Stawski, D.L. Goldstein, S. Durink, A. De Rienzo, Z. Modrzan, F. Nadin, T.T. Nguyen, B.S. Jaishwal, L.R. Chrysler, D. Sciarangella, N. Dao, C. E. Gustafson, J.K. Munir, J.A. Hackney, A. Chaudhuri, R. Gupta, J. Giulilly, K. Toy, C. Ha, Y.J. Chen, J. Sinnot, A. Chaudhuri, N. Zhang, T.D. Wu, D.J. Sugarbaker, F. J. de Sauvage, W.G. Richards, S. Seshagiri, Comprehensive genomic analysis of malignant pleural mesothelioma development and cooperate to accelerate tumorigenesis, PLoS One 6 (4) (2011), e18228.

[5] T. Hida, M. Hamasaki, S. Matsumoto, A. Sato, T. Tsuchiya, K. Kawahara, A. Iwasaki, T. Okamoto, O. Oda, H. Honda, K. Nabeshima, BAP1 immunohistochemistry and p16 FISH results in combination provide higher confidence in malignant pleural mesothelioma diagnosis: ROC analysis of the two tests, Pathol. Int. 66 (10) (2016) 563–570.

[6] F. Galateau-Salle, L. Jiang, H. Nagai, Y. Okazaki, H. Murakami, Y. Sekido, E. Arai, Y. Kanai, O. Hino, T. Takahashi, H. Nakagama, S. Toyokuni, Fenton reaction induced cancer in wild type rats recapitulates genomic alterations observed in human cancer, PLoS One 8 (12) (2013), e43043.

[7] S. Toyokuni, Novel aspects of oxidative stress-associated carcinogenesis, Antioxidants Redox Signal. 8 (7-8) (2006) 1373–1377.

[8] S. Toyokuni, Novel aspects of oxidative stress-associated carcinogenesis, Antioxidants Redox Signal. 8 (7-8) (2006) 1373–1377.

[9] K. Donaldson, F.I., M.C. Copin, S. Dacic, C. Danel, A. Foulet-Roge, A. Gibbs, S. Giusiano-Courcambeck, K. Hiroshima, V. Hofman, A. Husain, K. Kerr, T. Takahashi, S. Toyokuni, Homozygous deletion of CDKN2A/2B is a hallmark of iron-induced high-grade rat mesothelioma, Lab. Invest. 90 (2010) 360–373.

[10] S. Toyokuni, The origin and future of oxidative stress pathology: from the recognition of carcinogenesis as an iron addiction with ferritoxicity resistance to non-thermal plasma therapy, Pathol. Int. 66 (2016) 245–259.

[11] M. Hiroo, M. Otsuka, K. Koda, M. Ichihara, Y. Sekido, T. Tanaka, H. Hatai, S. Toyokuni, Specific allelic loss of p16 (INK4A) tumor suppressor gene after weeks of iron-mediated oxidative damage during rat renal carcinogenesis, Am. J. Pathol. 160 (2) (2002) 416–429.

[12] S. Toyokuni, Role of iron in carcinogenesis: cancer as a ferrotoxic disease, Cancer. Res. 57 (2007) 56–58.

[13] L. Jiang, H. Nagai, H. Ohara, S. Misawa, H. Yasui, S. Toyokuni, Deferasirox induces mesenchymal-epithelial transition in crocidolite-induced mesothelial carcinogenesis in rats, Cancer. Prev. Res. 6 (2013) 1222–1230.

[14] Y. Ohara, S.H. Chew, T. Shiibata, Y. Okazaki, K. Yamashita, S. Toyokuni, Phlebolomyctomy as a preventive measure for crocidolite-induced mesothelioma in male rats, Cancer. Sci. 109 (2) (2018) 330–339.

[15] T.D. Oury, T.A. Sporn, V.L. Roggli (Eds.), Pathology of Asbestos-Associated Diseases, third ed., Springer, Berlin/Heidelberg, 2014.

[16] M. Gay, L. Diao, D.L. Gibbs, V. Thorsson, L. Iype, H. Kantheti, D.T. Severson, U. Osmanbeyoglu, Y. Sekido, J. Takeshita, Y. Newton, K. Graim, M. Gupta, C. Ha, J. de Sauvage, W.G. Richards, S. Seshagiri, Comprehensive genomic analysis of malignant pleural mesothelioma identifies recurrent mutations, gene fusions and splicing alterations, Nat. Genet. 48 (4) (2016) 407–416.

[17] C. Zenklusen, R. Akbani, A.D. Cherniack, L.A. Byers, M.S. Noble, J.A. Fletcher, A. H. Pass, H. Kindler, M.G. Zauderer, D.J. Kwiatkowski, R. Bueno, A.S. Tsao, J. de Sauvage, W.G. Richards, S. Richards, Comprehensive genomic analysis of malignant pleural mesothelioma development and cooperate to accelerate tumorigenesis, PLoS One 6 (4) (2011), e18228.
H. Schagger, Tricine-SDS-PAGE, Nat. Protoc. 1 (1) (2006) 16

T. Hirayama, Fluorescent probes for the detection of catalytic Fe(II) ion, Free Radic. Biol. Med. 133 (2019) 38–45.
malignant mesothelioma in an orthotopic mouse model, Oncotarget 9 (26) (2018) 18494–18509.

[72] M.J. Brookes, J. Boult, K. Roberts, B.T. Cooper, N.A. Hotchin, G. Matthews, T. Iqbal, C. Tselepis, A role for iron in Wnt signalling, Oncogene 27 (7) (2008) 966–975.

[73] H. Yang, L. Pellegrini, A. Napolitano, C. Giorgi, S. Jube, A. Preti, C.J. Jennings, F. De Marchis, E.G. Flores, D. Larson, I. Pagano, M. Tanji, A. Powers, S. Kanodia, G. Gaudino, S. Pastorino, H.I. Pass, P. Finton, M.E. Bianchi, M. Carbone. Aspirin delays mesothelioma growth by inhibiting HMGB1-mediated tumor progression, Cell Death Dis. 6 (2015) e1786.