Epigenetic: A missing paradigm in cellular and molecular pathways of sulfur mustard lung: a prospective and comparative study

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ABSTRACT

Sulfur mustard (SM, bis-[2-chloroethyl] sulphide) is a chemical warfare agent that causes DNA alkylation, protein modification and membrane damage. SM can trigger several molecular pathways involved in inflammation and oxidative stress, which cause cell necrosis and apoptosis, and loss of cells integrity and function. Epigenetic regulation of gene expression is a growing research topic and is addressed by DNA methylation, histone modification, chromatin remodeling, and noncoding RNAs expression. It seems SM can induce the epigenetic modifications that are translated into change in gene expression. Classification of epigenetic modifications long after exposure to SM would clarify its mechanism and paves a better strategy for the treatment of SM-affected patients. In this study, we review the key aberrant epigenetic modifications that have important roles in chronic obstructive pulmonary disease (COPD) and compared with mustard lung.

Introduction

Cellular and molecular mechanisms of sulfur mustard

Sulfur mustard, bis (2-chloroethyl) sulfide, (SM), is a lethal chemical warfare agent (CWA), with high absorbance and alkylating potential that can affect tissues such as the lungs, eyes and skin. Also, it can be distributed through blood and systemically affect other tissues and organs, especially liver, brain, spleen, platelets, kidney, and white and red blood cells, (1). SM can directly interact with DNA bases including 7- (2-hydroxyethylthioethyl) guanine (7-HETE-G) (2), position 3 of adenine and O6 position of guanine (3) (Figure 1). Several repair pathways including poly (ADP-ribose) polymerase (PARP) pathway, base excision repair, nucleotide excision repair, non-homologous and joining are activated following SM exposure (4). SM exposure activates PARP pathway indicating its direct/indirect genotoxic effect, and also activates the intracellular repair system. Simultaneously, accumulation of p53 could block cell cycle and provide a time for up-regulation of repair proteins such as DNA polymerase b, stimulating of base excision repair (BER). After binding to DNA, PARP-1 synthesizes a poly (ADP-ribose)chain that is recruitment signals for other repair enzymes (5). Currently, it is proposed that PARP may be a switcher between apoptosis and necrosis (6), and may have regulatory function over apoptosis (7). If damage is not repairable, apoptosis will be followed and PARP will be cleaved. But if cell misses its energy

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Figure 1. DNA cross-linking by sulfur mustard
Figure 2. Overview of the molecular and cellular effects of sulfur mustard

SM (Sulfur Mustard); GADD45 (Growth Arrest and DNA Damage-inducible 45); PCNA (proliferating cell nuclear antigen); XRCC1 [X-ray repair complementing protein 1]; PARP (Poly (ADP-ribose) polymerase); IL (Interleukin); TNF-α (Tumor necrosis factor); TNFR-1 (tumor necrosis factor receptor-1); MCL1 (Monoclonal antibody cytokine protein 1); CCL2 (C-C motif chemokine 2); MMP9 (Matrix metalloproteinase 9)

HAT (histone acetyltransferase); CBP (CREB-binding protein); PcoG (Polycomb Group protein); PRC1 and PRC2 (Polycomb Repressive Complexes 1 and 2); HMT (histone methyltransferase); HUL (histone ubiquitin ligases); MBD (Methyl-CpG-binding domain); RAT (remover acetyltransferases); HDAC (Histone deacetylase); HDM (histone demethylases)

sources due to high ATP consumption of repairing system, necrosis will occur (8). Severe ATP depletion blocks cleavage of PARP by caspase-3 that leads to continuous activity of PARP (9).

In vitro and in vivo studies show some similarities between SM pathophysiology and other diseases such as chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and bronchiolitis obliterans (BO), but the exact pathophysiology of SM is not yet well understood (10). It was first proposed that acid liberation and hydrolysis of SM to HCl are the main causes of its toxicity. At the next step, glutathione depletion, protein inactivation, lipid peroxidation, and oxidative stress were postulated as mechanisms of SM toxicity (11, 12). To date, it is clear that SM alkylates nucleotides and causes intermolecular nucleotide cross-links, which is followed by genotoxic stresses and proteins or genome modifications. Moreover, SM interferes with natural function of proteins via misfolding, protein oxidation, antioxidant depletion, and cross-linking such as hexokinase inactivation. Also, lipids are peroxidized when exposed to SM (lipid peroxidation). Likewise, free radicals will be released as byproducts of lipid peroxidation. It is supposed that the
first and direct effect of SM exposure is oxidative stress, which is followed by arrest of cell signaling pathways and cell membrane collapse.

Innate immunity is the first defensive layer against toxic agents. Epithelial cells and macrophages are the primary layer of cells that can be exposed to SM in the pulmonary system. Following SM exposure, intense cellular and molecular alterations occur in lung. During several days after exposure, innate immunity induces adaptive immune system with pro-inflammatory mediators. If the apoptosis and necrosis rate increase, cell contents will be released into the extracellular matrix (ECM), and immune cells will be activated. Epithelial cell detachment, cell death, fibrosis, DNA repair system activation, tissue repair induction and systemic signaling are reported after SM exposure. Figure 2 shows the effects of SM and molecular and cellular alterations induced by SM in normal cell that described our current knowledge of SM induced cellular and molecular modifications.

**Oxidative stress and inflammation**

Oxidative stress has been detected in cell and tissue damages after misbalancing in physiological condition (13). It is also linked with many chronic inflammatory lung diseases such as asthma, COPD, IPF, OB, and adult respiratory distress syndrome (ARDS) (14). Oxidative stress has been implicated in the pathogenesis of SM exposure via unidentified mechanisms (15-18). Reactive oxygen species (ROS) are created in organisms after stimulation with biochemical hazardous stimulants molecules. There are several sources of exogenous and endogenous oxidants, such as smoking, ozone, pollutants, ionizing radiation, alcohols, peroxisomes, and phagocytes, of which cytochrome P450 enzymes, and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidases) are the most important endogenous sources of ROS production (19, 20). ROSs can be divided into two groups: free radicals and non-radical compounds. Free radical is an atom, molecule or compound, which is unstable and tends to interact with non-radical atoms, or unpaired electrons. ROS may alter the remodeling of apoptosis, extracellular matrix, mitochondrial respiration system, maintenance of surfactant, cell proliferation, the anti-protease screen, effective alveolar repair responses and immunity modulation in lung (21, 22).

Superoxide anion (O$_2^{-}$) is produced under respiratory burst, high energy requirements and increased respiration (23). The ROS can be transformed into highly toxic and stable substances through iron mediating reactions (24). Besides, nitric oxide (NO) is a strong natural oxidant that inhibits mast cell degranulation and histamine release. Despite NO is a strong oxidant, but ROS reacts with O$_2^{-}$ and forms a stable oxidant known as proxynitrite (ONOO$^-$), which can interact with bio-molecules and induce more damages (25-28). NO competes with enzymatic antioxidants in O$_2^{-}$ consumption, as well, high NO concentration disrupts enzymatic antioxidant equilibration. Moreover, SM depletes blood, hepatic and pulmonary glutathione (GSH) and increases its oxidized form (GSSG). Decrease in GSH content leads to the accumulation of naturally produced ROS within cells (17, 29). ROS causes mitochondrial damage and dysfunction that can lead to apoptosis (30). Accordingly, the role of ROS as a second messenger is accepted in four ways: degradation by particular enzymes, regulated enzymatic production, presence at low concentrations that can be transiently elevated upon stimulation, and facility to react at specific sites, for instance with metals and thiolates (31). Several studies have shown that these four characteristics can be attributed to ROS induced by SM (18, 32, 33).

SM exposure triggers several signaling pathways that result in inflammatory cytokine secretion such as TNF-α from alveolar macrophages, IL-6, IL-8, and GM-CSF (34, 35). SM causes widening of intercellular spaces and cell-matrix adhesion loss; therefore, mucus secretion is increased as cilia cannot beat them up. In patient with high SM doses exposure, rarely cilia on epithelial cells are observed and intracellular vacuoles are enlarged. Mucin (Muc5Ac) is also increased in these patients. There is a relation between the regulation of inflammation and alveolar macrophages, surfactant protein-1 (SP-D) and alveolar type II epithelial cell in SP-D production. After SM exposure, SP-D is decreased drastically (18). Extracellular proteases (released from injured cells, dead cells and immune cells) and oxidants cause tissue destruction and remodeling in SM lung. Normally, there is an imbalance of protease/antiprotease and oxidant/antioxidant pathways in SM vesicants (36, 37).

More exactly, lipid peroxidation, protein and nucleic acid alkylation, mutation, DNA breakage and repair, immune system induction and activation, injury sensing by neighboring cells, and systemic tissue repair systems are all reported as known pathways linked with SM acute injury (7, 38, 39). Newly released free radicals, depletion of antioxidants, cell content release of dead cells, unneutralized cellular ROS and RNS (reactive nitrogen species), along with immune system activation and inflammation will intensify the first step of oxidative stress (40). It is concluded that oxidative stress and inflammation induced by SM are two key factors that must be controlled for better treatment of SM intoxication. (Figure 2).

**Epigenetics**

**Introduction**

The epigenetics was first proposed by Waddington CH in 1940s (14, 41). Epigenetics describes all
meiotically and mitotically heritable changes in gene expression states that do not depend on DNA sequence (22, 42, 43). The epigenetic profile of a cell often dictates cellular differentiation and fate, as well as development, aging, disease and cancer (44-52).

Specific epigenetic modifications

Specific epigenetic modifications are classified into five general categories: DNA methylation (53), post-translational histone (54), noncoding RNAs (47, 50, 55-60), chromatin remodeling (ATP dependent chromatin remodeling complexes (CRCs)), histone variants (Histones with varying stabilities or specific domains). All of these modifications lead to gene activation or inactivation.

These modifications have been proceeding by three classes of bio-machines; a writer to create modifications on DNA and histone. A reader decipher codes and finally an Eraser to eliminate alterations (58, 61, 62) (Figure 3). In this paper, we focused on key modifications i.e. DNA methylation, histones modifications and noncoding RNA that have important roles in COPD as an inflammatory respiratory disease and compared with SM lung.

**DNA methylation**

DNA methylation is the most popular modification in DNA levels that occurs approximately in 3% of whole genome of eukaryotic cell. DNA methylation occurs largely on CpG islands that are more found in genes upstream (53, 63). Methylation of CpG islands interferes with binding of transcription factors and then suppresses all forums of genes expression (63, 64), especially developmental genes, repetitive sequences and germ-line specific (imprinted genes) (65, 66). DNA methylation catalyzes transfer of a methyl group from S-adenosyl methionine (SAM) to a cytosine residue to create 5-methyl cytosine (5 mC).

This process occurs by family of closely related DNA methyl transferases (DNMTs) as a writer (DNMT1, DNMT3a, and DNMT3b) (67). The readers of methylated DNA are methyl-CpG-binding domain proteins including Kaiso, MeCP2, and members of the methyl CpG-binding domain (MBD) family (66, 68). DNA demethylation could be passive or active. Active DNA demethylation occurs via direct removal of a methyl group. Active DNA demethylations such as MBD2b (methyl-CpG-binding domain protein 2b) (69), ten–eleven translocation (Tet) enzymes Tet1, Tet2, and Tet3 (70, 71), and AID/APOBEC (activation-induced cytidine deaminase/ apolipoprotein B mRNA-editing enzyme complex) are the most important eraser bio-machines in DNA modifications (72-74). The passive process takes place during replication of newly synthesized DNA strands by DNMT1. Base excision repair machinery (BER), and nucleotide excision repair (NER) are important passive DNA demethylation; however, there are many questions about the mechanisms of this bio-machines (75-77).

**Post-translational histone modifications**

The smallest unit of chromatin, the nucleosome, consists of 146 bp DNA sequence wrapped around a histone octamer (two copies of H2A, H2B, H3 and H4) linked by exterior histone H1. This organization guaranteed a close or tight structure to chromatin (78).
Different histone modifications are correlated with different functions on the lysines (K) and arginines (R)-rich tail region of histones. The H3 and H4 have a critical regulatory role in many diseases (79-81). There are many post-transcription or histone modifications, including acetylation (K), phosphorylating (P), methylation (M), citrullination, ubiquitination (Ubi), butyrylation, simulation, ADP-ribosylation, propionylation, and glycosylation of residues in the N-terminal tails of histones (Table 1) (82, 83).

Histones are acetylated by histone acetyltransferases (HAT). The acetylated histone marks H3K4ac and H3K39ac (as important marks of acetylation) are associated with transcriptional activation (84). The acetyl groups are removed by histone deacetylases (HDACs) that represses the gene activation by creating a tightly closed chromatin structure (78, 85, 86).

In contrast to acetylation, histone methylation can correlate either with transcriptional activity or inactivity. The histone methylation exists in three forms of mono, di and tri-methylation (87). By contrast, histone methylations on lysines 9 and 27 (H3K9me3 and H3K27me3) transcriptionally inactivate regions, broadly the whole gene, but more commonly at facultative heterochromatin (54, 88). The lysines (K) and arginines (R)-rich regions are methylated by histone methyl transferase enzyme (HMTs) and are removed by histone demethylases (HDMs) such as members of the Jumonji protein family (66, 67).

**Noncoding RNAs**

Noncoding RNAs are a class of small, mid-sized and long RNAs, which include the microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), and long noncoding RNAs (lncRNAs). Noncoding RNAs are hereditary involved in regulating the genes expression (89-91). miRNAs (19-24bp) play role in controlling transposable elements and direct DNA methylation at transposable elements. Each mature miRNA may target many genes and involve in development, differentiation, and cancer (92). Piwi-interacting RNAs are ~24-35 nt in length that involve in silencing transposable elements in the germ line and stem cell. These RNAs are involved in directing

**Table 1.** Histone modification post-transcription modification

| Modification types | Residue(s) modified | Reader domain(s) |
|--------------------|--------------------|-----------------|
| Unmodified lysine  | Lysine             | PHD             |
| Acetylation        | Lysine             | BRD             |
| Methylation        | Lysine/Arginine    | Ankyrin, Chromo, MBT, PHD, Tudor, PWWP, WD40 |
| Phosphorylation    | Serine/Threonine   | 14-3-3, BIR, BRCT |
| Ubiquitination     | Lysine             | BRD             |
| Sumoylation        | Lysine             | ?               |
| ADP-Ribosylation   | Lysine             | Tudor           |
| Glycosylation      | Serine/Threonine   | ?               |
| Butyrylation       | Lysine             | ?               |
| Propionylation     | Lysine             | ?               |

PHD (Plant Homeo domain); BRD (bromodomain); MBT (malignant brain tumor); BIR (Inhibitor of Apoptosis (IAP) family of proteins); BRCT (BRECA1 C’Terminus (BRCT) domain)
DNA methylation at more loci than just transposable elements and mutations in human. PIWI proteins are associated with infertility. Different PIWI proteins have non-redundant roles (93, 94). IncRNAs (>200 nt) are expressed in a controlled manner and are able to regulate epigenetic processes such as X inactivation, genomic imprinting, and DNA damage response. It was recently shown that rRNAs and piRNAs provide a dynamic balance between gene activation and silencing (95, 96).

**Epigenetic modification caused by inflammation in COPD**

Chronic inflammation is a main characteristic of COPD patients with GOLD stages I-III that related with activation of the NF-kB signaling pathway (97). Several studies have been conducted on aberrant epigenetic modifications and respiratory diseases (44, 45, 55). Intra- and extracellular ROS and NOS lead to a various range of pathophysiological conditions, as well as inflammation and oxidative stress (79). On the other hand, the fundamental role of inflammation and early aging in the development of COPD has remained unknown (98, 99). Recent articles have reported that inflammatory genes are regulated by transcription factors of the NF-kB, FOXP3, IRF, and STAT families, DNA methylation, and histone modifications (98, 100). Gene expression regulation is not only restricted to post-translational modification (PTM) of histone, but also is regulated by acetylation, methylation, phosphorylation, and ubiquitylation. More studies have investigated enzymes involved in this process. For example, the relation of HAT/HDAC enzymes has been evaluated in many respiratory diseases, especially in corticosteroid resistances (101-103). In Figure 4, some important functions of HAT/HDAC ratio are shown. Study of HAT/HDAC ratio in induction of pro-inflammatory proteins such as NF-kB and Ap-1 is very important and necessary that can control many inflammatory processes and signaling in the cell. Acetylation is an important modification in autoimmune and inflammatory diseases such as Th17-Threg balancing (104-106). So, the writer (HAT) and eraser of acetylation (HDAC) are studied in diseases, such as asthma, COPD, cancer, autoimmune disease, etc (107, 108).

In Table 2, some important epigenetic changes are listed that involved in the process of inflammatory diseases. Several reports have also demonstrated alterations in histone proteins in COPD. As a result, it must be considered in oxidative stress and inflammation induced by SM as well as diseases such as asthma and COPD (102, 108, 109). HDAC activation due to the effect of SM may lead to anti-inflammatory proteins and antioxidant enzymes silencing (109). HDAC-2 is the most important protein from HDAC families, which plays a role in inflammation and oxidative stress of lung disease. In a study, Barnes et al (86) proposed that stimulants such as nitric oxide and superoxide dismutase reduced HDAC-2 levels in COPD and asthma patients who eventually cause corticosteroids resistance (108, 110-112). Depending on the cause, the pathway is different and each pathway of HDAC-2 is reduced eventually (113, 114). Blocking the inflammatory gene expression pathway by inhibiting DNMT is the logical therapeutic approach in inflammatory diseases (85, 109).

**SM and possible epigenetic modifications in lung**

Several pathophysiological studies on SM exposed patient have shown significant imbalances and alterations in inflammatory mediators. This variation reflects the inflammatory roles of SM in the chronic phase (18, 115-117). Pro-inflammatory cytokines such as IL-1α, -8, -6, -13 (34), TNF-α (35), IFN-α (34, 118), GM-CSF (119) and stress induced proteins i.e. HSP 27, -70, -90 (120), SWI/SNF, iNOS, and MIP-1 (121) are trace in serum and tissue samples of SM patients. Regarding the inflammatory role of SM, it seems, nearly all epigenetic cods for expression of pro-inflammation proteins can be altered in epithelial and immune cells. These alterations consist of hypo and hyper methylation of CpG islands, histone modifications, long noncoding RNA expression, and chromosome remodeling.

Moreover, some clinical manifestations and diseases for example, COPD (33), lung cancer (1, 122, 123), and chronic bronchitis (124, 125) have been reported post exposure to toxic inhalators such as SM. This data propose that SM could induce epigenetic changes in cells and tissues (126). The pathophysiological similarities between pulmonary fibrosis and SM-induced lung toxicity, as well as bronchiectasis (125, 127) has been determined by previous studies (119, 127, 128). The pathogenesis of fibrosis is influenced by aberrant epigenetic modifications. Most of the demethylations occur in promoter regions of different genes encoding autocrine growth and differentiation factors of fibroblast cells (129). Therefore, it is speculated that SM-induced toxicity may be mediated by epigenetic perturbations at least in lung tissue, demonstrating a need to investigate protease/anti-protease imbalance in mustard lung (109).

Methylation of tumor suppressor genes leads to activation of oncogenes that promotes growth factor-independent proliferation of fibroblasts. Expression of miR-21 is an example of this type of aberrant modifications that leads to the degradation of tumor suppressor genes in fibroblasts (129). So, it is necessary to measure the expression of micro-RNA and DNA methylation of promoter in bronchoalveolar lavage (BAL) and tissue of mustard lung (130). Future molecular studies will be recognized the pathophysiological mechanism of this disease. Increased acute phase reactant proteins in SM exposed patients, for instance amyloid A1 and haptoglobin have been reported in studies of Mehrani
et al (33). In another study, Shahriay et al showed that in addition to inflammation, a protease/anti-protease imbalance exists as well. In this way, endoplasmic reticulum (ER)-60 protease, S100 A9, serpin B1, and glutathione-S-transferase were significantly altered (33).

Several key epigenetic modifications are recognized in repair and remodeling pathways of airway inflammatory lung diseases. Some of these important changes are selective inhibition of INOS, cyclooxygenase-2, and MMPs, especially the MMP-9 (147). MMP-9 is epigenetic cross-talk and interfering protein in suppression of NF-κB cascade pathways or p300-HAT expression within the nucleus (148). Similarly, Norani et al have shown that metallothioneins (MTs) (149) and SODs (150) are higher in the control group compared to SM-exposed groups. This can be due to oxidative stress pathways as a result of mustard gas toxicity in airway wall of SM exposed patients. Hypermucosal secretion is another pathophysiologic problem of mustard lung patients (18, 126, 149). As a prospective investigation, the study of epigenetic modifications like the DNA hypomethylation of SMD or MMPs (32) can be a novel molecular explanation and therapeutic guideline for mustard lung (151).

It seems the complexity of post SM exposure such as inflammation, oxidative stress, protease/anti-protease imbalance, should be resolved epigenetically view; in particular, DNA methylation and tissue-specific patterns. Despite, a few data are currently available regarding the possibility of an epigenetic basis for the effects of SM, some evidences have shown the potential role of downregulation of pro-inflammatory genes, alterations in histone modifications, and gene
expression changes in chromatin regulatory enzymes as potentially epigenetics modifications in chronic phase of SM (40, 81).

The Figure 5 listed the possible epigenetics change in NF-kB signaling pathway. For instance, increasing of ATP remodeling increasing the HAT activity (for induction of pro-inflammatory proteins), and increasing H4k9 and H4k16 acetylation (for secretion of pro-inflammation factors) can be important epigenetic modification. Hypermethylation of repetitive genes, and CpG poor promoters and locus specific DNA hypomethylation in p52-RelB protein binding site are some of the DNA modification. Finally, all this modification can trigger pro-inflammation factors such as IL-1β, -8, -6, -13, TNF-α and iNOS (152).

**Future directions in research area of SM**

Overall, our previous study highlights that SM generally stimulates inflammation and oxidative stress pathways in chronic phase (153). Our findings suggest that molecular mechanisms of SM intoxication in gene expression occur independently

![Figure 5. Sulfur mustard and possible epigenetic modifications in chronic phase. p53 MAPK (p53 Mitogen-activated protein kinase kinase 3); NF-kb (nuclear factor kappa-light-chain-enhancer of activated B cells); Ap-1 (Activator protein 1); HAT (histone acetyltransferase); IL (interleukin); TNFα (tumor necrosis factor alpha); Inf-α (Interferon alpha); HSP 27 (heat shock protein 27); p53 MAPK (p53 Mitogen-activated protein kinase kinase 3); iNOS (Inducible nitric oxide synthase); MIP-1 (Macrophage Inflammatory Proteins 1); GM-CSF (Granulocyte-macrophage colony-stimulating factor)
of changes in the DNA sequence (32, 33). These epigenetic mechanisms include PTM of nucleosomal histone, DNA methylation, and regulation by noncoding RNAs (154, 155). Here, we discussed the relationship between the latest achievements in this area with epigenetics through biology systems approach. The reversible epigenetic modifications are prospect therapeutic key of inflammatory airway diseases such as COPD. Evidently, to hypothesize that epigenetic changes play a role in inflammation of COPD (88), oxidative stress (113, 121), and other inflammatory disorders (10, 156), it is necessary to clarify what causes the epigenetic changes. On the other hand, it has been proved that the patterns of cellular and molecular changes of SM poisoning are similar to such diseases (157). Data presented here suggest precise mechanism of SM poisoning to achieve the correct perspective in diagnostics or therapies (157, 158). At the clinical level, molecular studies will help us to find out the pathogenesis of SM lung and effective molecular treatment. The HDACi or cyclooxygenase inhibitors can be the main treatment options for SM-lung patients (158). The study of whole genome and epigenetics modification, as well as next generation assays may give an integrated view of the unique integrative framework for the complexity and pathologic diversity of SM.

Concluding remarks

In summary, several epigenetic modifications occur in lung inflammatory diseases such as COPD, asthma, IPP, and even in SM animal models that these changes trigger the complex molecular pathways such as NF-kB, PARP, Jack-Stat, inflammation, proteins signaling pathway, mitochondrial metabolism, and oxidative stress. To achieve the missing link between clinical and molecular findings and effective molecular therapies, these epigenetics modifications should be reviewed more in depth as a biological system.

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