International Immunopharmacology 61 (2018) 37–44

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

DNA damage and telomere length shortening in the peripheral blood leukocytes of 20 years SM-exposed veterans

Hossein Behboudi[a], Sakineh Kazemi Noureini[b], Tooba Ghazanfari[c], Sussan K. Ardestani[a,*]

[a] Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran
[b] Department of Biology, Faculty of Basic Science, Hakim Sabzevari University, Sabzevar, Iran
[c] Immunoregulation Research Center, Shahed University, Tehran, Iran

ARTICLE INFO

Keywords:
Sulfur mustard
Oxidative DNA damage
Cellular senescence

ABSTRACT

Sulfur mustard (SM) is a vesicant chemical warfare agent, and a very potent alkylating agent. SM exerts its cytotoxicity via direct alkylation of biomacromolecules, and overproduction of reactive oxygen species (ROS). Previous studies have shown that SM-induced oxidative stress has adverse effects on antioxidant defense system, and damages lipids and proteins. The aim of this study was to investigate the effect of SM-induced oxidative stress on DNA damage, and cellular senescence in SM-exposed victims. For this purpose, MDA levels as a measure of oxidative stress in the serum, 8-oxo-dG content of the genomic DNA, and OGG1 expression as two biomarkers of oxidative DNA damage, as well as, telomere length, and $p16^{INK4a}$ expression as two biomarkers of cellular senescence were measured in the peripheral blood leukocytes of 215 males who were exposed to SM 20 to 25 years ago, and 53 unexposed healthy males as the control group. Our results indicated that the levels of 8-oxo-dG, and OGG1 mRNA expression were significantly higher in SM-exposed individuals. Furthermore, a significant increase in the expression of $p16^{INK4a}$ was observed in SM-exposed patients, and leukocyte telomere length (LTL) was also significantly shorter in severe/very severe cases of SM-exposed patients when compared with unexposed controls. In conclusion, our data indicate that oxidative DNA damage is higher in SM-exposed patients, and their immune system has subjected to cellular senescence.

1. Introduction

1-Chloro-2-(2-chloroethylsulfanyl) ethane or sulfur mustard (SM) is a vesicant chemical warfare. World War I, and the Iran-Iraq war (1980–88) are the two major incidences that SM has been used extensively as a chemical weapon. In Iran-Iraq military conflict more than 100,000 Iranian individuals were exposed to SM, and over 30,000 victims are still suffering from its long-lasting toxic effects [1]. SM early effects on the human body have been investigated, but there are scarce information about the long-term effects of SM on exposed victims. Skin, eyes, and respiratory system are the primary targets for SM. However, the respiratory system is more susceptible to long-term complications. Chronic Obstructive Pulmonary Disease (COPD), pulmonary fibrosis, chronic bronchitis, bronchiectasis, bronchiolitis obliterans, large airway narrowing, and asthma are the main late pulmonary complications in SM-exposed individuals. The underlying molecular, and cellular mechanism of SM long-term toxicity is not clear, however, the SM toxicity has been largely attributed to its ability in alkylating macromolecules such as lipids, proteins, and DNA. Forming adducts, and inactivation of these macromolecules inhibits nucleic acid, protein, and ATP biosynthesis which in return leads to the disruption of cellular energy metabolism, and uncovered production of reactive oxygen species (ROS) [2,3]. Previous studies have shown that SM induces oxidative stress through overproduction of endogenous ROS, suppressing cell's antioxidant defense system, and oxidative DNA repair [4,5]. Signs of increased oxidative stress have also been observed in SM-exposed Iranian victims in several studies. For instance, the level of low molecular weight antioxidants such as glutathione (GSH) has been shown to significantly decrease in their serum while a significant increase in the malondialdehyde (MDA) level has been observed in the serum and Bronchoalveolar lavage (BAL) fluids of SM-exposed individuals [6,7]. Furthermore, alterations in the expression and activities of some antioxidant enzymes have also been observed [7–10]. In addition to the direct genotoxic effect of SM, the endogenous oxidative stress induced by SM through cellular signaling and inflammation also damages DNA. Accumulation of these oxidative DNA lesions may cause gene expression modifications, genetic mutations, and chromosome instability that all together result in impairment of cell growth, apoptosis, and tissue damage [5,11]. It has been suggested that oxidative stress has an important role in modulating telomere length [12]. Telomeres are

* Corresponding author.
E-mail address: ardestany@ut.ac.ir (S.K. Ardestani).

https://doi.org/10.1016/j.intimp.2018.05.008
Received 31 January 2018; Received in revised form 13 May 2018; Accepted 14 May 2018

repetitive sequences of 5′-TTAGGG-3′ with 3′ overhangs at the ends of linear chromosomes. The single stranded 3′ overhang is created due to the inability of DNA polymerase α to replicate the very end of the chromosome. Telomeres with the aid of a series of proteins known as shelterin create nucleoprotein complexes which turn the telomeric DNA into a loop structure known as the T loop. These complexes cap and protect the chromosome's ends from nuclease degradation, end to end fusion, and being recognized as double strand breaks, and therefore, play a critical role in maintaining genome integrity and stability. In somatic cells, there is no significant telomerase activity, and telomeres shorten inevitably in each round of cell division due to end replication problem [13]. Beside this, other contributing factors such as oxidative stress which is usually created in an inflammatory state may accelerate telomere shortening [14]. The high content of guanine in telomere sequence makes it exceptionally sensitive to reactive oxygen species. Hydroxyl radicals oxidize all four bases of DNA, however, guanine exhibit the lowest ionization potential, which makes it the most susceptible DNA target for oxidation reaction [15]. 8-Oxo-2′-deoxyguanosine (8-oxo-dG) is an oxidized derivative of deoxyguanosine, and is the major oxidative DNA lesion created by ROS. 8-Oxoguanine glycosylase (OGG1) is a DNA glycosylase enzyme responsible for the removal of 8-oxo-dG in the base excision repair process [16,17]. The action of OGG1 creates single strand breaks in the DNA, which are poorly repaired in telomere sequences, and may lead to loss of greater telomere stretches in each round of replication [15]. Progressive attrition of telomere, and dissociation of the shelterin components activate DNA damage response (DDR) which may triggers apoptosis or irreversible growth arrest. Following the DDR response or other stressors, p21, and $p16^{INK4a}$ cyclin-dependent kinase (CDK) inhibitors are overexpressed, and arrest cells at the G1 phase of the cell cycle. In this state cells are metabolically active but are unable to proliferate, and termed senescent cells. $p16^{INK4a}$ is expressed in response to various stresses such as DNA damage, Telomere dysfunction, and oncogenic signaling, and blocks inappropriate cellular division via inhibiting CDK 4/6 which results in hypophosphorylation of pRB, and subsequent inactivation of E2F1 transcription factor, and eventually blocking the transition from G1 to S phase of the cell cycle [18]. $p16^{INK4a}$ is highly expressed in senescent cells, and it has been suggested as a biomarker of cellular senescence [19].

In this study, in order to evaluate oxidative DNA damage in SM-exposed veterans, we measured 8-oxo-dG content, and OGG1 gene expression in their peripheral blood leukocytes. In an effort to investigate the effect of SM exposure on immune system aging, and cellular senescence, we also measured leukocyte telomere length and leukocytes $p16^{INK4a}$ gene expression in SM-exposed victims.

2. Material and methods

2.1. Study population

The study was performed on 215 Iranian male individuals who were exposed to sulfur mustard gas 20 to 25 years before sample collection in 2008. All SM-exposed patients had developed clinically significant late pulmonary complications. 53 healthy male members of the patient's families with similar age distribution, and residence who were not exposed to SM and didn't have any pulmonary complications were selected as the control group. At the time of sample collection, a medical history of diabetes, hypercholesterolemia, hypertension, and smoking status was obtained from all participants by trained medical staff. All the patients were consented under the direction of Iranian Foundation of Martyrs and Veterans Affairs review board. Prior to admission to the study, an informed consent was obtained from all participants. Spirometry was performed on all the participants according to the American Thoracic Society Criteria with spirometry device (Chest 801 Spirometry) under the supervision of a well-experienced nurse at the time of sample collection.

2.2. Sample collection

15 ml Fasting venous blood was collected from each participant in Na_2EDTA coated tubes, and aliquots of whole blood and serum were kept under liquid nitrogen until later use.

2.3. Serum malondialdehyde (MDA) measurement

Serum MDA level was measured based on TBARS assay (Thiobarbituric acid reactive substances) as describes by Satoh [20]. Briefly, to 0.1 ml of fasting blood serum, 0.2 ml trichloroacetic acid (20%) was added, and centrifuged at 2000*g* for 10 min. The pellet was then washed once with 0.5 ml 50 mM sulfuric acid and heated in a boiling water bath for 30 min at the presence of TBA reagent (0.2% in 2 M sodium sulfate) and 50 mM sulfuric acid. The resulting lipid peroxide adducts were subsequently extracted by *n*-Butanol, and absorbance was measured at 530 nm. The concentration of MDA was calculate by using the standard curve created by MDA standard solution (1,1,3,3-tetraetoxypropane in 50 mM H_2SO_4), and the results were reported as μmol/l.

2.4. DNA extraction

Genomic DNA was extracted from 10 ml whole blood using salting out procedure with minor modifications [21]. Since the extracted DNA was intended to use for quantification of leukocytes 8-oxo-dG content, in order to prevent accidental ROS generation, and DNA damage, DNA extraction lysing buffer was treated with 1 mM ascorbic acid and 1 mM deferoxamine (an iron chelator). Extracted DNA was quantified by using Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and samples with 260/280 absorbance ratios ≥1.8 were considered as pure DNA. DNA integrity was also checked by running samples on 0.8% agarose gel.

2.5. DNA oxidation measurement

For evaluating DNA oxidative damage, the 8-oxo-dG content of the leukocytes genomic DNA was measured by 8-oxo-dG detection Elisa kit. For this purpose, initially 5 μg of extracted DNA was digested with nuclease P1 (Sigma, N8630) to release nucleotides, and then dephosphorylated by treating with alkaline phosphatase (NEB, M02903). Prepared samples were subsequently subjected to 8-Oxo detection ELISA kit (Cayman chemicals, 589320) in a total volume of 50 μl according to manufacturer's protocol. All samples were run in duplicates, and 8-oxo-dG concentrations were reported in pg/ml.

2.6. Leukocyte telomere length (LTL) measurements

Relative mean telomere length was measured using Monochrome Multiplex qPCR (MMqPCR) method, also known as Cawthon method, as described previously [22]. In brief, 20 ng of DNA was quantified in triplicate on a Rotor-Gene Q 6plex real-time PCR Platform (Qiagen, Germany) in a total volume of 20 μl with final concentrations of 1× HOT FIREPol® EvaGreen® HRM Mix (Solis Biodyne, Korea), 0.9 μM of telc, and telg primers for telomere sequence amplification, and 0.9 μM of albu, and albd primers for the albumin as a single-copy gene. The primer sequences are shown in Table 1. PCR cycling condition was as follow: 1 cycle, 15 min at 95 °C; 2 cycles, 15 s at 95 °C, and 15 s at 49 °C; 35 cycles, 15 s at 94 °C, 10s at 62 °C, 15 s at 74 °C (with signal acquisition), 10 s at 84 °C, 15 s at 88 °C (with signal acquisition). Five serial dilutions of a reference DNA (pooled from five controls) spanning 1.85–150 ng were also run in triplicates on each assay to generate standard curves. The ratio (T/S) of the telomere repeat number (T) to the single copy gene Albumin number (S) of each sample was determined by the standard curve method. T/S, therefore, is a relative, and dimensionless value which is proportional to the average telomere