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Mustard gas exposure and carcinogenesis of lung

Alireza Hosseini-khalili[a,b,*], David D. Haines[a], Ehsan Modirian[b], Mohammadreza Soroush[b], Shahriar Khateri[b], Rashmi Joshi[a], Kazem Zendehdel[c], Mostafa Ghanei[d], Charles Giardina[a]

[a] Department of Molecular and Cell Biology, University of Connecticut, USA
[b] Janbazan Medical and Engineering Research Center, Iran
[c] Cancer Research Center, The Cancer Institute, Tehran University of Medical Sciences, Iran
[d] Research Center for Chemical Injuries, Baqiyatallah University of Medical Sciences, Tehran, Iran

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ABSTRACT

Sulfur mustard (SM), also known as mustard gas, is an alkylating compound used as a chemical weapon in World War I and by Iraqi forces against Iranians and indigenous Iraqi Kurds during the Iran–Iraq War of the 1980s. Although SM is a proven carcinogen there are conflicting views regarding the carcinogenicity of a single exposure. The present study characterizes lung cancers formed in mustard gas victims from the Iran–Iraq War.

Methods and materials: Demographic information and tumor specimens were collected from 20 Iranian male lung cancer patients with single high-dose SM exposures during the Iran–Iraq War. Formalin-fixed, paraffin-embedded lung cancers were analyzed by immunohistochemistry for p53 protein. In addition, DNA was extracted from the tissues, PCR amplified and sequenced to identify mutations in the p53 and KRAS genes associated with SM exposure.

Results: A relatively early age of lung cancer onset (ranging from 28 to 73 with a mean of 48) in mustard gas victims, particularly those in the non-smoking population (mean age of 40.7), may be an indication of a unique etiology for these cancers. Seven of the 20 patients developed lung cancer before the age of 40. Five of 16 cancers from which DNA sequence data was obtainable provided information on eight p53 mutations (within exons 5–8). These mutations were predominately G to A transitions; a mutation consistent with the DNA lesion caused by SM. Two of the lung cancers had multiple p53 point mutations, similar to results obtained from factory workers chronically exposed to mustard agent. No mutations were detected in the KRAS gene.

Discussion: The distinguishing characteristics of lung carcinogenesis in these mustard gas victims suggest that a single exposure may increase the risk of lung cancer development in some individuals.

1. Introduction

The alkylating compound sulfur mustard (SM) has been employed as a chemical warfare agent since its first use in World War I [1]. The most recent application of this weapon was during the Iran–Iraq conflict by the Iraqi Baathist government. During this war, which lasted from 1980 to 1988, Iraqi dictator Saddam Hussein made frequent use of SM as a battlefield force multiplier and also extensively targeted unprotected civilians in Iran and within the Kurdish regions of Iraq [2]. Over 50,000 survivors of SM attacks remain alive in Iran and as a group suffer from high rates of chronic illnesses, particularly inflammatory conditions of the respiratory system, skin and eye [3–5], which are the major target organs of SM. Latent respiratory syndromes include asthma, chronic bronchitis, bronchiectasis, bronchiolitis, bronchial stenosis, and sinusitis [3,4]. The low cost and ease with which SM may be manufactured, transported and deployed, along with negligible odor and ability to cause permanent injuries after a few seconds exposure have made it historically the most frequently used chemical weapon during military operations.

A particularly insidious feature of SM has also increased its potential as a combat force multiplier: tissue damage due to the agent typically does not appear for 16 h or more after exposure. Hence civilians and Iranian troops with often heavy SM exposure, would assume that absence of symptoms after light, or no decontamination meant that the danger had passed. Such victims

* Corresponding author at: Molecular & Cell Biology, BSP 305, 91 North Eagleville Road, Unit 3125, Storrs, CT 06269-3125, USA. Tel.: +1 860 486 0089; fax: +1 860 486 4331.
E-mail address: alireza_hosseini50@yahoo.com (A. Hosseini-khalili).
URL: http://www.mcb.uconn.edu/fac.php?name=giardinac (A. Hosseini-khalili).

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typically developed symptoms within a day, often full-body blistering, loss of most skin, deep tissue injury and organ failure [2]. These characteristics also make future battlefield and terrorist use of this chemical a real threat. Nevertheless, there are few comprehensive studies on the long-term health consequences of SM exposure, a gap in medical knowledge with potential for significantly adverse impact on public health management of future mass casualties.

The enormous pool of Iranian SM victims, with carefully documented exposure and medical histories, therefore offer an unparalleled resource and opportunity for developing a mechanistic understanding of SM-associated chronic health problems. In the present report we examine selected clinical, pathological and genetic features of Iranian SM victims suffering from lung cancer as an element of a broader investigation to assess the potential carcinogenic risk associated with an acute SM exposure.

The long-term effects of SM exposure may develop as a result of two major processes. One pathway leading to chronic illness in SM victims occurs due to a failure of host immunoregulatory mechanisms to resolve inflammation triggered by the episulfonium ion. Here, high levels of inflammatory cytokines inhibit apoptosis of polymorphonuclear leukocytes, particularly neutrophils, thus prolonging their active state and amplifying damage done by these cells [6,7]. A second major pathway leading from SM exposure to disease can occur when the compound causes mutations in tumor suppressor and oncogenes, such as p53 or KRAS. SM is a known alkylating agent, and under conditions of chronic exposure, is a recognized carcinogen [8]. The mechanism of SM-induced carcinogenesis begins with cyclization of SM in the aqueous environment of a victim, to a highly reactive episulfonium ion which may alkylate DNA. If these are not repaired, these lesions can lead to nucleotide substitutions [9], most commonly the G to A transition [10]. This mutation may inactivate tumor suppressor genes such as p53, and greatly increase susceptibility to lung cancer, as was observed in Japanese mustard gas factory workers chronically exposed to SM [11–13]. Although chronic SM is a known carcinogen [14–17], there are conflicting views regarding the carcinogenicity of a single exposure [18–21]. From a public health perspective, acute exposure is a much more relevant exposure scenario in either past and potential future military conflicts or terrorist attacks.

Studies of cancer incidence in survivor populations with single, high-dose SM exposures have thus far failed to demonstrate strong correlations between exposure and disease occurrence. Bullman and Kang followed the outcome of US veterans exposed to SM in World War II [21]. Although analysis of these patients showed no significant increase in lung cancer risk, exposure incidences were relatively low and smoking habits were not considered. There is no study in the medical literature addressing the affect of a single, high-dose SM exposure on the long-term risk of lung cancer. Now, after two decades, Iranians with well-documented exposures to mustard agent in the 1980s can be studied to help clarify the lung cancer risk associated with a battlefield exposure to SM.

Multiple lines of evidence will be required before a causative link between acute mustard agent exposure and lung cancer development can be established. Ongoing epidemiological studies are being pursued in Iran to determine if mustard-exposed individuals are at greater risk for lung cancer development, but these efforts will require an extended period of time before a sufficient number of lung cancer cases develop, and may take years to complete. For the present study, we have collected data from Iranian SM victims with unambiguous, documented exposure histories, who eventually developed lung cancer. Records from the Iran–Iraq war also provide information on the precise exposure timeframes during the 8-year conflict. Here, specific personal attributes of victims and molecular/genetic features of their cancers were examined to evaluate carcinogenic effect of SM exposure on lung tissue. These studies include a mutational analysis of two tumor suppressor genes: p53 and KRAS, both of which are known indicators of gene–environment interactions impacting cancer risk. In summary, our data support the view that a single exposure to mustard agent may trigger cancer development in some individuals.

2. Methods and materials

2.1. Patient population

The present study was conducted using medical record data provided by Janbazan Medical and Engineering Research Center (JMERC) and archived paraffin-embedded lung tumor samples from pathology departments of major Iranian medical centers treating persons exposed to chemical weapons during the Iran–Iraq war. Here, data and samples were drawn from a subject population of 20 Iranian males with single, battlefield SM exposures resulting in acute and chronic symptoms including skin and respiratory injuries. All exposures occurred in the years 1982–1988 and subjects were subsequently diagnosed between the ages of 28 and 73 with three major forms of lung carcinoma (Table 1). Subjects were randomly selected from among deceased individuals for whom complete records and samples existed. Time intervals between exposure to the weapon and onset of disease ranged from 5 to 20 years. Subjects included 5 current or former smokers, 9 non-smokers and 6 with indeterminate histories of tobacco use (Table 1). We were unable to assemble a significant number of matched tumor samples from patients without a history of SM exposure and smoking that were preserved in a manner that allowed DNA extraction. We therefore used International Agency for Research on Cancer (IARC) database as our baseline for p53 mutational frequencies in lung tumors [8].

2.2. Mustard exposure, inclusion and exclusion criteria

Mustard exposure in this study is defined as any contact with SM in liquid or vapor form, resulting in transient or permanent disability. This definition is based on standards developed in a comprehensive national survey accomplished during the timeframe 1997–2000 that established a uniform convention for designation of Iranian citizens with war-related chemical injuries. Under this convention, mustard exposure is defined as any contact with SM in liquid or vapor form, resulting in transient or permanent disability. Here the minimum threshold for SM-induced disability is defined according to known primary effects of the agent on its major target organs: eyes, skin and lungs. Threshold exposure definitions for each organ are as follows: eye: edema and visible inflammation of ocular membranes; skin: redness accompanied by obvious blistering; and lung: edema accompanied by inflammation and either a productive cough, or hemoptysis in the form of bloody streaks or expectoration of clots. These criteria take into account the highly variable length of time and concentration ranges of SM that personnel are typically subjected to under battlefield conditions and make no attempt to correlate SM dosage with symptoms. Some estimation of SM dosage sustained by subjects of this study may nevertheless be estimated based on reference ranges of the agent known to produce particular outcomes. Acute exposure guideline levels (AEGLs) for SM have been developed by the U.S. National Advisory Committee (NAC). Exposure to SM at 0.60 milligrams/cubic meter (mg/m^3) of air for 10 min; or 0.013 mg/m^3 for 8 h constitute the threshold level at which edema of the eyes, sensitivity to light, and eye irritation occur [22]. These ocular symptoms also define the threshold level for SM exposure established by Janbazan organization. Therefore participants in this study were exposed to at least the level of SM identified by the NAC as needed to produce critical ocular symptoms. Patients participating in this study were selected on the basis of documented exposure to SM based on official certification from the Iranian Veteran's Affairs organization (Janbazan). This documentation included records of medical treatment showing the type and extent of mustard-associated injury and/or disability. Patients with histories of serious major disease other than lung cancer were excluded from this study. This investigation was conducted under the approval of Janbazan organization's ethics committee.

2.3. Tissue preparation and DNA extraction

Lung cancer tissue was obtained from biopsies or surgical sample, fixed with formalin and embedded in paraffin. Neoplastic lesions from representative areas of 4–5 unstained slides containing 10 μm thick tissue slices were scraped into a microcentrifuge tube. After paraffin removal with xylene, tissues were re-hydrated and the DNA was isolated using the PicoPure™ DNA extraction Kit (Arcturus Engineering Inc., Mountain View, CA), according to the manufacturer's instructions.

2.4. DNA amplification and sequencing

The extracted DNA was amplified by the polymerase chain reaction (PCR), using a nested primer approach. The primer sets for p53 exons 5, 6, 7 and 8 are shown in Table 2. Initial amplification reactions yielded the target amplimer with a number of off-target products. The specific product was then selectively amplified using nested p53 primers positioned a few bases down-stream of the initial primers. The