The American Journal of Pathology, Vol. ■, No. ■, ■ 2016

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

Molecular Mechanism Underlying Pathogenesis of Lewisite-Induced Cutaneous Blistering and Inflammation

Chemical Chaperones as Potential Novel Antidotes

Q31 Changzhao Li,* Ritesh K. Srivastava,* Zhiping Weng,* Claire R. Croutch,† Anupam Agarwal,* Craig A. Elmets,* Farrukh Afaq,* and Mohammad Athar*

Q2 *From the Departments of Dermatology and Medicine,* University of Alabama at Birmingham, Birmingham, Alabama; and the Medical Countermeasures Division,† MRIGlobal Research Institute, Kansas City, Missouri*

Accepted for publication June 21, 2016.

Address correspondence to Mohammad Athar, Ph.D., Department of Dermatology, University of Alabama at Birmingham, 1530 3rd Ave. S., VH 509, Birmingham, AL 35294-0019. E-mail: mathar@uab.edu.

Lewisite is a potent arsenic-based chemical warfare agent known to induce painful cutaneous inflammation and blistering. Only a few modestly effective antidotes have so far been described in the literature. However, the discovery of effective antidotes for lewisite was hampered by the paucity of the exact molecular mechanism underlying its cutaneous pathogenesis. We investigated the molecular mechanism underlying lewisite-induced cutaneous blistering and inflammation and describe its novel antidotes. On the basis of our initial screening, we used a highly sensitive murine model that recapitulates the known human pathogenesis of arsenicals-induced cutaneous inflammation and blistering. Topically administered lewisite induced potent acute inflammation and microvesication in the skin of *Ptch1*$^{+/-}$*/SKH-1* mice. Even at a very low dose, lewisite up-regulates unfolded protein response signaling, inflammatory response, and apoptosis. These cutaneous lesions were associated with production of reactive oxygen species and extensive apoptosis of the epidermal keratinocytes. We confirmed that activation of reactive oxygen species–dependent unfolded protein response signaling is the underlying molecular mechanism of skin damage. Similar alterations were noticed in lewisite-treated cultured human skin keratinocytes. We discovered that chemical chaperone 4–phenyl butyric acid and antioxidant *N*-acetylcysteine, which significantly attenuate lewisite-mediated skin injury, can serve as potent antidotes. These data reveal a novel molecular mechanism underlying the cutaneous pathogenesis of lewisite-induced lesions. We also identified novel potential therapeutic targets for lewisite-mediated cutaneous injury. *(Am J Pathol 2016, ■: 1–13; http://dx.doi.org/10.1016/j.ajpath.2016.06.012)*

Q6 Lewisite [dichloro (2-chlorovinyl) arsine] is a potent arsenical vesicant chemical warfare agent with significant systemic toxicity.[1] It was first synthesized in 1904 and later rediscovered by Captain W. Lee-Lewis in 1918 in the United States. Although it was proposed for use as a chemical weapon, fortunately it was never applied to the battlefield.[2] Nonetheless, it is known that several countries, including Germany, Italy, the United States, Russia, and Japan, have stockpiled significant amounts of lewisite,[3] causing a significant concern for public health. Being

Supported by NIH National Institute of Arthritis and Musculoskeletal and Skin Diseases CounterACT Program grants R21 AR064595 and UO1 NS095678 (M.A.). Q3 Q4

C.L. and R.K.S. contributed equally to this work.

Disclosures: None declared.

NIH had no involvement in the study design; collection, analysis, and interpretation of data; the writing of the manuscript; or the decision to publish the manuscript.

A guest editor acted as the editor-in-chief for the manuscript. No one at University of Alabama at Birmingham was involved in the peer review process or final disposition. Q5

http://dx.doi.org/10.1016/j.ajpath.2016.06.012

highly toxic and quick-acting vesicant chemicals, lewisite and other structurally related arsenicals, such as methyldichloroarsine, phenyldichloroarsine, and ethyldichloroarsine, have always been considered to be potential candidates for chemical weapons.[1] Unintentional exposure or intentional use by terrorists could be another significant threat. Other analogs of lewisite, such as phenylarsine oxide (PAO), phenydiidoarsine, *trans*-chlorovinylarsine oxide, and *trans*-chlorovinyldiiodide, manifest similar but less severe effects in murine skin and human skin xenograft.[4]

Lewisite can burn and blister any part of the body it comes in contact with. It is considered much more reactive than mustard gas.[5] If not decontaminated effectively and immediately, an individual can be killed by 30 drops (2.6 mg) of lewisite exposed to the skin.[2] The death may be caused by its systemic toxicity from lewisite shock, which is severe fluid loss and hypovolemia secondary to capillary leakage.[6] Besides skin, eyes and respiratory tract are the most likely targets of lewisite.[2] The physicochemical characteristics of lewisite that include its lipophilic nature make it penetrate the skin rapidly. Topically exposed lewisite induces acute inflammation associated with severe pain, which develops within 10 to 12 seconds, followed by erythema, edema, and blistering, which appear later.[7] Its reactivity with glutathione, leading to its loss followed by a decrease in overall protein thiols, was considered the major mechanism for these manifestations. Dysregulation of calcium homeostasis due to oxidative stress, lipid peroxidation, and membrane damage, leading to cell death, is also described.[1]

Early strategies that led to the development of British anti-Lewisite (BAL) as its antidote are based on the arsenic chelating properties of BAL. Indeed, BAL treatment had some efficacy in reducing lewisite-induced tissue damage.[8] However, BAL is a toxic compound that has very low solubility in water. Its treatment requires painful intramuscular injections.[9] Water soluble analogs of BAL have less toxicity compared with BAL but reduced efficacy against lewisite-induced skin lesions.[9] Thus, other than BAL, largely there is no effective US Food and Drug Administration–approved therapeutic approach to reduce lewisite toxicity. Therefore, developing novel, more efficacious therapeutic drugs for counteracting lewisite-induced toxicity will largely depend on defining its molecular pathogenesis.

Endoplasmic reticulum (ER) is the site of biosynthesis, assembly, folding, and maturation of many secretory and membrane-bound proteins. Disruption of ER homeostasis may result in the accumulation of unfolded and/or misfolded proteins, leading to the condition known as ER stress. On ER stress, unfolded protein response (UPR) signaling is activated. The UPR pathway regulates the biosynthesis of chaperone proteins. These chaperone proteins bind with partially folded or unfolded proteins to restore the protein-folding capacity of ER and provide a balance between protein-folding overload and impaired ER capacity. This is achieved by engaging three ER membrane resident proteins: PERK, IRE1, and ATF6. However, prolonged activation of UPR signaling may lead to the pathogenesis of inflammation and tissue damage by inducing cell death. UPR-regulating proteins have also been associated with multiple other conditions, such as neurodegenerative and metabolic disorders and tumorigenesis.[10]

The interplay of reactive oxygen species (ROS) and ER stress is also found under certain experimental conditions.[11] Because at least some of the systemic effects of lewisite are thought to be related to arsenic toxicity, in this study we explored the mechanism by which lewisite induces acute cutaneous inflammation and tissue damage. We believe that arsenic could play an important role in the manifestation of lewisite toxicity. Our data indeed indicate that topical challenge of lewisite onto the skin of *Ptch1*$^{+/-}$*/SKH-1* mice and treatment of human keratinocytes with lewisite activate the UPR-signaling pathway, inflammatory responses, and cell death, suggesting a role of UPR signaling in lewisite-mediated tissue injury. Interestingly, treatment with the chemical chaperone 4-phenylbutyric acid (4-PBA) afforded protection against lewisite-induced inflammation and tissue injury by blocking the UPR-signaling pathway. These data strengthen the role of this pathway in lewisite toxicity. We also found upstream involvement of ROS in triggering lewisite-mediated skin damage. Consistently, the observation that the antioxidant *N*-acetylcysteine (NAC) affords significant protection against lewisite toxicity suggests that ROS-regulated UPR signaling is one of the underlying key molecular pathways of lewisite cutaneous toxicity. Therapeutic approaches targeting UPR signaling and ROS may lead to the development of novel and highly effective antidotes against lewisite-induced tissue damage.

Materials and Methods

Materials

Lewisite was synthesized by MRIGlobal Research Institute (Kansas City, MO). PAO (P3057), NAC (A7250), and 4-PBA (P21005) were from Sigma-Aldrich (St. Louis, MO). CM-H2DCFDA (C6827) was from Life Technology (Carlsbad, CA). Immortalized human keratinocytes, HaCaT cells (T0020001), was from AddexBio Technologies (San Diego, CA). SSoFast Eva Green Supermix (172-5202) was from Bio-Rad (Hercules, CA). Antibodies cleaved caspase-3 (9664), phospho-eIF2α (3398s), eIF2α (9722), CHOP (2895), ATF4 (11815s), phopho-JNK1/2 (9251), and phospho-c-Jun (9261) were from Cell Signaling (Danvers, MA); GRP78 (sc-1050) and phospho-IκBα (sc-101713) were from Santa Cruz (Dallas, TX); phospho-NF-κB p65 (ab30623) and IL-1β (ab9722) were from Abcam (Cambridge, UK); cyclooxygenase 2 (COX2) (160126) was from Cayman Chemical (Ann Arbor, MI); F4/80 (14-4801-82) was from eBioscience (San Diego, CA); and CD11b (BD557395) and Gr1 (BD553126) were from BD (San Jose, CA). Cignal Finder 45-Pathway Reporter Array plate (CCA-901L), RT^2 First Strand kit (330401), RT^2 qPCR Master

FLA 5.4.0 DTD ■ AJPA2412_proof ■ 12 August 2016 ■ 5:20 pm ■ EO: AJP16_0212