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Sulfur mustard downregulates iNOS expression to inhibit wound healing in a human keratinocyte model

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siRNA

Summary

Background: Increased nitric oxide (NO) synthesized by inducible NO synthase (iNOS) is involved in inflammatory and pathological conditions. iNOS also regulates several biomarkers that accelerate normal wound healing. Effects of exposure to sulfur mustard (SM) on the skin include formation of blisters and slow-healing injuries. Promoting re-epithelialization is a challenging issue in the treatment of the delayed healing of SM-induced skin injuries.
Objectives: To clarify the role(s) of iNOS in wound healing and the effect of SM on iNOS expression in an *in vitro* wound assay to eventually develop therapies for SM skin injuries.
Methods: A wound was created by scratching normal human epidermal keratinocytes grown *in vitro*. iNOS expression was monitored by Western blotting, fluorescence microscopy, and real-time RT-PCR. Wound healing was analyzed using digitalized image analysis software.
Results: The level of iNOS peaked 24—48 h after wounding. SM exposure strongly reduced iNOS protein and mRNA levels. Fluorescence microscopy revealed that induction of iNOS expression by wounding and inhibition of iNOS expression by SM occurred not only in the cells at the wound edge but also in cells in the surrounding area, suggesting that wounding may induce and SM may inhibit release of cytokines that stimulate iNOS expression. iNOS-specific small interfering RNAs caused a marked decrease of iNOS expression irrespective of wounding. Gene silencing also completely inhibited wound healing.

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Conclusion: These results suggest that preventing SM-induced inhibition of iNOS may be a prospective strategy to promote wound healing in SM-exposed skin.

1. Introduction

Sulfur mustard (SM) (2, 2′-dichlorethyl sulfide) is an alkylating agent, vesicant and chemical warfare agent that has been used militarily since World War I, including most recently by Iraq against Iran (1984—1987) [1]. The major target organs are the skin, eyes, and respiratory tract [2]. In the skin, SM produces chemical burns with widespread blistering that can lead to incapacitation, serious injuries and/or death of the victims depending on the conditions of the dose and/or duration of SM exposure. Wound healing from an injury due to SM exposure is considerably slower than for a comparable thermal burn. Despite extensive studies on the mechanisms of SM tissue injury [3], the precise mechanisms of the blistering and subsequent healing have not been elucidated. Because there is no effective antidote, the current standard therapy is palliative [4]. Accelerating the healing process of the SM-damaged skin has been a challenging problem but an important one to solve.

Nitric oxide (NO) is a short-lived molecule (half-life of 5—10 s) involved in both physiological and pathological processes of almost all organ systems [5]. This double-edged sword effect [6] is apparently created by the amount and timing of NO produced in the tissue. NO is synthesized from L-arginine by the gene products of four NO synthase (NOS) genes. Generally, low levels of NO are produced by the constitutively expressed, Ca^{2+}-dependent neuronal and endothelial NOSs (nNOS or NOS1 and eNOS or NOS3, respectively,) mostly for homeostasis in various tissues [7]. The third NOS, iNOS or NOS2, is inducible, Ca^{2+} independent, and is responsible for production of large amounts of NO in response to stimulation by cytokines or bacterial metabolites [8]. A fourth NOS is also Ca^{2+} dependent and named mitochondrial NOS (mtNOS), but most of its functions remain to be elucidated [9].

Skin consists of the epidermal and dermal layers, with the stratified keratinocytes and the collagen-rich dermis providing support and strength. *In vivo*, wound healing is carried out by many cell types, such as epidermal cells (keratinocytes), macrophages, fibroblasts, platelets and inflammatory cells at the injured site. Upon skin injury, all of these cells respond to the injury by producing various kinds of growth factors, cytokines, mitogens, and NO that can affect the healing process of the injured skin either positively, when released in an orchestrated manner, or negatively, when released in an uncoordinated manner [10]. Re-epithelialization carried out by keratinocytes is a critical step toward the healing process, and migration, proliferation and differentiation of keratinocytes in a concerted manner play important roles in restoring the normal architecture and function of the skin [11]. Recent evidence clearly indicates that NO produced by iNOS followed by NO-mediated signaling in the skin plays a pivotal role in skin wound healing [11]. However, the roles of NO in skin wound healing vary depending on the study model employed and/or the stage of wound healing observed. For example, suppression or deletion of iNOS, which is the major producer of NO in the skin, delays or arrests wound healing; however, in contrast, introduction of the iNOS gene to the wounded area rescues delayed wound healing in a mouse model [13,14]. On the other hand, NO production by iNOS suppresses regulated upon activation normal T-cell expressed and secreted (RANTES) [14], a necessary cytokine in an early stage of wound healing, suggesting that the role of NO in wound healing cannot simply be described as having either a positive or a negative effect on wound healing. Instead, its effect may depend on the stage of the healing process. Regardless, data published to date indicate that NO mainly produced by iNOS regulates wound healing by being balanced the expression of the major cytokines at the appropriate stage to sometimes enhance and sometimes inhibit iNOS expression to control normal wound healing [6].

In the present study, we hypothesized that iNOS expression is a critical factor for skin wound healing in normal human epidermal keratinocytes (NHEKs) and that SM exposure suppresses iNOS to inhibit wound healing in a NHEK wound healing model.

2. Materials and methods

2.1. Materials

NHEKs, keratinocyte growth medium (KGM: Epilife®), human keratinocyte growth supplement (HKGS), and antibiotic mixture (gentamicin and amphotericin B) were obtained from Cascade