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CRYSTAL ARTHRITIS (MH PILLINGER, SECTION EDITOR)

Uric Acid and Xanthine Oxidoreductase in Wound Healing

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Abstract

Chronic wounds represent a significant health issue as they are difficult to heal and treatment is often complicated, lengthy and expensive. For a majority of sufferers the most common outcomes are long-term immobility, infection and prolonged hospitalisation. Therefore, there is an urgent need for the development of effective therapeutics that will enhance ulcer healing rate, patient quality of life and reduce healthcare costs. Studies in our laboratory have demonstrated elevated levels of purine catabolites in wound fluid from patients with venous leg ulcers. In particular, we have discovered that uric acid is elevated in wound fluid with higher concentrations correlating with wound severity. We have also demonstrated a corresponding depletion in uric acid precursors, including adenosine. Further, we have shown that xanthine oxidoreductase, the enzyme that catalyses the production of uric acid, is present at elevated levels in wound fluid. Taken together, this provides evidence that xanthine oxidoreductase may play a role in the formation or persistence of chronic wounds. Here we describe the potential role of xanthine oxidoreductase and uric acid accumulation in the wound site and the effect of xanthine oxidoreductase in potentiating the inflammatory response.

Keywords

Uric acid; Xanthine oxidoreductase; Inflammation; Wound healing; Topical allopurinol

Introduction

Chronic leg ulcers affect 1-3% of adults aged over 60 years and while studies have shown that approximately 50% of leg ulcers heal within six months, many remain unhealed for years[1, 2].

Venous leg ulcers are the most frequently encountered chronic wounds in the clinical setting[3]. These wounds represent one of the biggest health issues as they are difficult to heal and treatment is often lengthy and expensive. Vulnerable individuals include those 70 years and over with impaired mobility, peripheral vascular disease and a sluggish reparative process[4]. Currently the most effective treatment strategy for venous leg ulcers involves the application of compression therapy to the affected limb. However, up to 30% of chronic venous leg ulcers do not respond to compression and remain unhealed, even after a year of treatment[5]. The impact of chronic wounds is expected to grow given the ageing population and the increased incidence of cardiovascular disease, diabetes and obesity.

Chronic venous leg ulcers are normally hypoxic in nature owing to poor tissue perfusion[6]. Hypoxia triggers a chain of events ultimately resulting in tissue damage that further impedes wound closure. This oxygen deficit and cell injury leads to the depletion of intracellular ATP and initiates the atypical build-up of purine metabolites[7]. Tissue stores of ATP are catabolised sequentially to adenosine monophosphate, inosine monophosphate, adenosine, inosine and hypoxanthine, resulting in an accumulation of these metabolites in tissue[7]. Xanthine oxidoreductase (XOR), a complex molybdo-flavoenzyme, is subsequently required for the conversion of hypoxanthine to xanthine, and finally to uric acid while liberating the toxic superoxide radical. We have previously published data that demonstrated a correlation between elevated uric acid in wound fluid and wound severity in a cohort of venous leg ulcer patients [8]. We also observed decreased levels of purines precursors (sum totals of adenosine, inosine, xanthine and hypoxanthine) in wound fluid collected from patients with more severe wounds[8]. Previous reports indicate that topical application of purine precursors accelerates wound healing in various animal and cell culture models[9-11]. In particular, adenosine has been shown to play an important role in stimulating wound healing[12, 13]. Further, we have shown that XOR, the only enzyme in humans capable of catalysing the production of uric acid, is present at elevated levels in wound fluid and correlates with wound severity[8]. Based on these novel findings, we believe the presence of elevated levels of XOR and uric acid may play an important part in sustaining inflammation in chronic wounds via three key mechanisms (Figure 1). These mechanisms include:

- Depleting the key precursor purines, inosine and adenosine, that have been shown to promote dermal wound healing[9-11];

- Increasing the amount of uric acid and urate crystals in the wound environment that could further stimulate the inflammatory response, much like gout; and
- Releasing excessive levels of reactive oxygen species at the wound site.

In this review, we will discuss the potential role of XOR and uric acid in delayed wound healing by prolonging the inflammatory response. We also propose that XOR may represent a suitable therapeutic target for chronic wounds. Inhibition of XOR using specific inhibitors, such as allopurinol, could simultaneously target three major contributors that keep chronic wounds in a non-healing state - uric acid accumulation, oxidative stress and purine precursor depletion.

Depletion of purine precursors at the wound site

Our data indicates an increase in purine precursors in wound fluid collected from patients with clinically less severe or healing wounds[8]. This is not surprising given that the purine precursor, adenosine, has been shown to play an important role in stimulating wound healing[12, 13]. Extracellular adenosine induces biological responses by interacting with one or more of the four adenosine cell surface receptors, A_1, A_{2A}, A_{2B} and A_3. Specifically, the activation of the A_{2A} adenosine receptor has also been reported to enhance the rate of wound healing in both healthy and diabetic murine wound models[9]. In addition, cells involved in wound healing including macrophages, fibroblasts and endothelial cells have been shown to express the A_{2A} adenosine receptor[14-16]. The role of the A_{2A} adenosine receptor in wound healing was later confirmed by Montesinos *et al.* 2002[10] using an A_{2A} adenosine receptor knockout mouse model. The lack of A_{2A} adenosine receptors in these transgenic mice resulted in disorganized granulation tissue and significantly lower blood vessel formation[10].

Adenosine has also been shown to be a potent regulator of inflammation[17]. Studies have reported that adenosine acts through its receptors to inhibit oxidative bursts and degranulation in neutrophils[18] and the release of cytokines from macrophages[19-21]. Adenosine also decreases leukocyte recruitment, inhibits neutrophil adhesion to the endothelium and neutrophil mediated endothelial damage [22-26]. Activation of adenosine receptors also contributes to the formation of granulation tissue and new blood vessel formation. The A_{2A} adenosine receptor suppresses the production of thrombospondon 1, an inhibitor of angiogenesis, enhancing vascular vessel formation[27]. Therefore, it is likely that the breakdown of adenosine as a result of oxygen depletion or cellular and tissue injury has the potential to alter wound healing processes. This is

supported by our data that demonstrates reduced concentrations of purine precursors in wound fluid from clinically severe, non-healing wounds. Thus, XOR activity may be related to wound severity due in part to increased catabolism and depletion of available adenosine.

Accumulation of uric acid stimulates the inflammatory response

The role of uric acid in many human inflammatory disorders remains unclear. Elevated levels of uric acid have been associated in heart disease, stroke, diabetes and more recently in wound fluid from patients with venous leg ulcers[28-30]. Hyperuricemia commonly leads to the formation of needle like monosodium urate (MSU) crystals that is dependent on factors including sodium levels, pH and temperature[31-33]. The inflammatory effects of uric acid, however, rely on the precipitation to MSU crystals and their recognition by immune cells, particularly mononuclear phagocytes. There have been a number of proposed mechanisms for MSU associated inflammasome activation including activation of TLR (toll like receptors) and sensing changes in intracellular potassium (Reviewed in Jin et al., 2012)[34]. The most recent MSU pathway reported is the activation of the NLRP3 inflammasome leading to the production of active IL-1β[35]. MSU crystals are engulfed by phagocytes resulting in lysosomal damage and rupture. The lysosomal contents are sensed by the NLRP3 inflammasome resulting in the activation of caspase 1, which processes proinflammatory cytokines such as pro-IL-1β and pro-IL-18 to their active forms[36, 37]. Of interest, previous studies have demonstrated elevated levels of IL-1β in wound fluid from patients with chronic wounds[38-40]. IL-1β production in response to MSU stimulation leads to increased recruitment of neutrophils to the site of inflammation[41]. This observation is in accordance with recent studies using inhibitors of IL-1 and animal models with defective IL-1β production[35, 42]. Neutrophils infiltrating an already inflamed site will be activated by MSUs. MSU-mediated activation releases large amounts of proinflammatory cytokines and ROS while delaying neutrophil apoptosis[43], which further exacerbates the inflammatory response[44].

A recent study demonstrated that uric acid levels in various organs are elevated following cell death[45]. Importantly, the study demonstrated that the depletion of extracellular and intracellular uric acid inhibits the inflammatory response triggered by sterile cell death. However, this effect was not observed in the presence of microbial molecules or sterile irritants *in vivo*[45]. This indicates that uric acid specifically promotes an inflammatory response as a result of cell death[45]. Elevated levels of uric acid in the chronic wound environment are likely to prolong inflammation. Sustained production of uric acid in underperfused damaged tissues may lead to

the crystallisation of uric acid at the wound site, as observed in the case of gout[46]. Indeed, wound management clinicians have noted anecdotally the presence of such crystals in wounds. Thus, the accumulation of these crystals in an already inflamed area may therefore provide added stimulus, further intensifying the inflammatory response.

Effect of elevated levels of free radicals on the wound environment

Uric acid has been proposed to act as an antioxidant by scavenging metal ions and oxidants, especially peroxynitrite[47-49]. The action of these oxidising agents on uric acid leads to the formation of allantoin despite the lack of the specific enzyme (urate oxidase) in humans[50]. However, the production of uric acid itself is associated with a burst of the toxic superoxide free radical which can elicit cellular damage[51]. The accumulation of uric acid at the wound site suggests that XOR is also present and active, oxidising hypoxanthine, as well as xanthine, while liberating reactive oxygen species (ROS).

There is growing evidence to suggest that ROS are involved in the pathogenesis of chronic venous leg ulcers[52]. This damage is initiated by toxic superoxide radicals that are generated by a several enzyme systems, including NADPH oxidases and XOR[53, 54]. Superoxide itself is unstable[53] and is rapidly converted to H_2O_2, either spontaneously or enzymatically by superoxide dismutase[55-57]. H_2O_2 readily diffuses across cell membranes, combining with metal ions like iron, and generating the toxic hydroxyl radical[58-60]. Hydroxyl radicals are highly reactive, resulting in the oxidation of cellular components[61, 62]. A wound environment rich in oxidants may activate redox-sensitive transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1)[57, 63-65]. The activation of these transcription factors up-regulates various genes that are involved in the production of pro-inflammatory cytokines, including matrix metalloproteinases (MMP)[61, 66]. In addition to the classic oxidants, activated phagocytes secrete myeloperoxidase (MPO), a degrading heme peroxidase that catalyses the oxidation of H_2O_2 to form HOCl[67, 68]. At low concentrations this potent oxidant possesses the ability to activate latent MMPs, in particular MMP-7, -8 and -9[69, 70], which we have previously shown to correlate with wound chronicity[71]. The perpetual expression of pro-inflammatory cytokines, exacerbated proteolytic activity and the decrease in levels of growth factors are believed to be key factors underlying wound chronicity.

Free radicals have a variety of harmful effects at both the cellular and tissue levels. An imbalance of ROS at a cellular level leads to an exacerbated inflammatory response resulting in the expression of various proinflammatory cytokines such as interferon (IFN), tumour necrosis factor-α (TNF-α) and interleukins (ILs); these have been shown to regulate the expression of XOR[72-75]. At the tissue level, redox-associated modification of protein thiol groups by ROS, such as H_2O_2, can give rise to a series of intermediate products[76]. Modification of cysteine residues may lead to the reversible formation of mixed disulphide bonds within or between protein thiol groups, as well as low molecular weight thiols[77]. Similarly, HOCl can modify free sulfhydryl groups leading to the formation of disulfide bonds that can cause irreversible protein aggregation *in vivo*[78, 79]. Oxidative modification of thiol groups could alter protein structure, impairing protein function, and effecting downstream redox signalling pathways[80]. These modifications could potentially decrease cell proliferation, vascularisation and prevent re-epithelialisation, resulting in prolonged healing times. Therefore, we believe that XOR is an overlooked source of ROS production and to some extent may play an important part in prolonging the inflammatory process through the liberation of the superoxide radical.

Topical allopurinol as a treatment for chronic wounds

Chronic wound care accounts for 3% of the total healthcare expenditure in developed countries[81]. However, the total costs to society is the sum of these direct costs plus indirect costs associated with loss of productivity, the immeasurable psychological cost of pain and diminished quality of life. The demand for wound care is expected to rise over the next decade due to an increase in chronic wounds as a direct result of lifestyle related disorders such as cardiovascular disease, obesity and diabetes. While there have been vast improvements in community care and a surge in the variety of wound dressings available, venous ulcers remain a challenge to treat, highlighting the need for improved therapies to improve healing rates. This is largely due to the poor understanding of the mechanisms that underlie this condition. Management of chronic venous leg ulcers is subjective, predominantly relying on the clinician's expertise in the field. Indeed, this is compounded by the limited treatment options and the absence of specific molecular or biochemical tests to guide clinical decision-making.

Our data indicating enhanced turnover of purine precursors in clinically worse ulcers supports the notion that XOR may represent a novel therapeutic target. Allopurinol is a potent inhibitor of

XOR and is primarily used in the treatment of gout and hyperuricemia[82, 83]. It is a registered medication (>30 years) and is delivered orally at 200 to 300 mg for patients with mild gout and up to 900 mg for those with moderately severe tophaceous gout[84]. A number of studies have also reported positive results with the use of topical allopurinol in the treatment of corneal alkali burns[85] and in patients with radiation-induced mucositis and dermatitis[86]. Allopurinol is therefore an ideal candidate for inhibiting XOR activity.

The accumulation of uric acid in the wound environment, as demonstrated by our preliminary data cannot be assessed in an animal model. Unlike humans many animals species including rodents and pigs express the enzyme uricase[87]. Uricase catalyses the conversion of uric acid to allantoin and therefore uric acid never reaches the critical concentrations as observed in the human disease, gout. Since no clinically relevant animal or *in vitro* models of chronic venous leg ulcers exist**,** we are currently assessing topical allopurinol in a Phase I safety trial on a subset of patients with chronic venous leg ulcers. We propose that topical allopurinol treatment will decrease the amount of uric acid and ROS released into the wound environment, while at the same time restoring depleted levels of critical purines such as adenosine and inosine. Allopurinol was chosen as the preferred inhibitor for future clinical trials in wounds as it is cheap, has low toxicity and can be easily monitored using its breakdown product oxypurinol. Topical application is the preferred mode of delivery as it is unclear if oral administration of the drug will effectively target the wound site due to the poor microcirculation caused by venous insufficiency in these patients. More importantly, topical application avoids the complications that high dosage systemic allopurinol could provoke in patients with multiple comorbidities as observed in a majority of patients presenting to clinics with chronic wounds. Ultimately, we aim to demonstrate that topical treatment with allopurinol will prevent sustained inflammation and stimulate wound repair, thus reducing the duration of compression therapy. Successful completion of this project will lead to the implementation of a targeted evidence-based therapeutic for treatment of these chronic recurring lesions.

Conclusions

The development of more effective, yet affordable, treatments is particularly important for wound care needs. In view of this, there is an urgent need for the identification and development of effective novel wound therapies that can be used in the clinical setting to better manage

patients with chronic wounds. Chronic wounds are characterised by an amplified and prolonged inflammatory phase. Given the nature of these wounds, we believe that inhibition of XOR will restore homeostasis at the wound site and enable damaged tissue to return to normal healing. We anticipate that the proposed treatment, when used in combination with routine high compression therapy, has the potential to reduce healing times, improve patient quality of life and reduce healthcare costs.

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Compliance with Ethics Guidelines

The Phase I safety trial is being conducted in accordance with the ethical guidelines of the Declaration of Helsinki and ICH Good Clinical Practice Guidelines. Prior to commencement of the trial, approval was obtained from the Therapeutic Goods Association (TGA) in Australian and the Queensland University of Technology Human Ethics Committee (Ethics Clearance number – 1200000635). The trial is also registered in the Australian New Zealand Clinical Trials Registry (ANZCTR) and allocated the ACTRN: ACTRN12613000568718. Written Informed consent is obtained from all patients before enrolment in to the study and study records are de-identified to maintain patient confidentiality.

Conflict of Interest

Melissa L. Fernandez is employed by and received grant support from the WMI CRC, has received grant support from the National Health and Medical Research Council, and has had travel/accommodations expenses covered/reimbursed by the WMI CRC and Institute of Health and Biomedical Innovation.

Zee Upton has received grant support from the National Health and Medical Research Council, has served as a consultant for Tissue Therapies Ltd. and Smith and Nephew, is a chief investigator on research grants from/involving Tissue Therapies Ltd., has applied for patents planned/filed through Tissue Therapies Ltd., has purchased stock in Tissue Therapies Ltd., has

had travel/accommodations expenses covered/reimbursed by WMI CRC, and has undertaken contract research for Novartis. In addition, her husband is an inventor of patents licensed to Tissue Therapies Ltd., consults for Tissue Therapies Ltd., has personally bought stock in Tissue Therapies Ltd., and is a chief investigator on grants from/involving Tissue Therapies Ltd..

Gary K. Shooter has received grant support from the National Health and Medical Research Council, has served as a consultant for Tissue Therapies Ltd., has had travel/accommodations expenses covered/reimbursed by the WMI CRC, and has worked on projects in which patent applications have been filed by the Queensland University of Technology.

Melissa L. Fernandez and Gary K. Shooter are also named inventors on the aspects of this project that has been patented by The Wound Management Innovation Pty.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Figure 1 – Proposed Mode of Action of XOR and Uric Acid at the Wound Site – The presence of elevate levels of xanthine oxidoreductase (XOR) and uric acid could play a role in sustaining inflammation at the wound site by (a) generating excessive amounts of ROS; (b) formation of MSU crystals; and (c) depleting key purine precursors that contribute to wound healing.