Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Nutritional Biochemistry 17 (2006) 328–336
Journal of Nutritional Biochemistry

Dietary supplementation of *N*-acetylcysteine enhances early inflammatory responses during cutaneous wound healing in protein malnourished mice☆

Yunsook Lim[a,b], Mark A. Levy[a], Tammy M. Bray[a,b,*]

[a]*Department of Human Nutrition, The Ohio State University, Columbus, OH, USA*
[b]*College of Health and Human Sciences, Oregon State University, Corvallis, OR 97331, USA*

Received 7 April 2005; received in revised form 9 July 2005; accepted 9 August 2005

Abstract

Prolonged wound healing is a complication that contributes to the morbidity and mortality of protein malnutrition (PM). The molecular mechanisms that underlie impaired wound healing in PM may begin in the early inflammatory stage of the process. We hypothesized that the impaired wound healing observed in PM occurs as a consequence of excessive reactive oxygen species (ROS) production that impairs the wound healing process by depressing nuclear factor kappa B (NFκB) activation and the subsequent synthesis and release of proinflammatory cytokines that are critical mediators of the inflammatory response. In this study, we showed that the time to wound closure was significantly prolonged in PM mice. During the early wound healing, inhibitory kappa B alpha (IκBα), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) expression and neutrophil infiltration were significantly decreased in PM mice. The role of excess ROS in PM was demonstrated by using transgenic mice with overexpression of copper zinc superoxide dismutase and with dietary supplementation of *N*-acetylcysteine (NAC). Both interventions improved the extent of wound closure in PM mice. Moreover, NAC supplementation in PM mice restored the expression of IκBα, IL-1β and TNF-α and infiltration of neutrophils to levels observed in control animals. These findings support the notion that wound healing defects in PM may result from dysregulation of ROS-mediated and NFκB-regulated signaling pathways.

Keywords: Wound healing; Protein malnutrition; Reactive oxygen species; N-acetylcysteine; NFκB

1. Introduction

Wound healing is an essential process that serves to repair and regenerate tissue structure and function that has been disrupted, or wounded, by physical, chemical, bacterial or viral insults. In general, there are three major stages of wound healing: inflammation, proliferation and remodeling [1]. Notably, the early stage of inflammation is regarded as a critical period of the wound healing process, essential for clearing the contaminating bacteria and creating an environment conducive to succeeding events involved in tissue repair and regeneration [2–4]. Inflammation at the wound site is marked initially by the infiltration of neutrophils. These are the predominant inflammatory cells during the early inflammatory stage that serve to prevent infection through phagocytic processes and propagate the inflammatory response by releasing cytokines and chemokines [4–6]. The recruitment and function of neutrophils have been shown to be regulated by several cytokines, including interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [1,6,7]. Both IL-1β and TNF-α expressions are regulated by nuclear factor kappa B (NFκB), a redox-sensitive transcription factor activated by reactive oxygen species (ROS) during periods of oxidative stress [8]. Hence, NFκB occupies a central position within the early inflammatory cascade [9,10], and NFκB activation is thus proposed to be a critical event of the wound healing process [11,12].

Cutaneous wound healing is a complex process involving inflammatory mediators and numerous genes that have been

Abbreviations: CuZnSOD, copper zinc superoxide dismutase; ROS, reactive oxygen species; GSH, glutathione, L-γ-L-glutamyl-L-cysteinyl-glycine; IκBα, inhibitory kappa B alpha; IL-1β, interleukin-1β; NAC, *N*-acetylcysteine; NFκB, nuclear factor kappa B; PM, protein malnutrition; TNF-α, tumor necrosis factor-α.

☆ This research was supported by the National Institutes of Health grant DK55847 and NS38315 to T.M.B. We are grateful to Dr. Ning Quan and his laboratory in the Division of Oral Biology, School of Dentistry, The Ohio State University, for the technical support for the histology assay and in situ hybridization.

* Corresponding author. College of Human and Health Sciences, Oregon State University, Corvallis, OR 97331, USA. Tel.: +1 541 737 3256; fax: +1 541 737 4230.
E-mail address: tammy.bray@oregonstate.edu (T.M. Bray).

0955-2863/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2005.08.004

only partially identified. This is particularly true of wound healing during periods of malnutrition, where recovery time and complications associated with wound healing are markedly increased. For example, protein malnutrition (PM), a major health issue that affects people in developing and developed countries [13–15], is characterized by prolonged and poor wound healing as well as depressed immune function and antioxidant defense [16–20]. Although the molecular mechanisms that underlie impaired wound healing in PM remain obscure, evidence from human studies indicates that the blood levels of specific cytokines, namely, IL-1β and TNF-α, are depressed, and the balance between ROS generation and antioxidant defense is disturbed in PM [21–24]; these factors may contribute to immunosuppression and subsequent complications associated with wound healing and tissue repair.

We hypothesized that the delayed wound healing observed in PM patients occurs as a consequence of excessive ROS production, which impairs the wound healing process by depressing NFκB activation and the subsequent synthesis and release of proinflammatory cytokines that are critical mediators of the inflammatory response. To test this hypothesis, we used transgenic mice that express different levels of copper zinc superoxide dismutase (CuZnSOD), a first line of defense antioxidant enzyme, and dietary supplementation of *N*-acetylcysteine (NAC), a cysteine prodrug that increases glutathione (GSH) levels, to examine the role of ROS during the early stage of cutaneous wound healing in a mouse model of PM. In particular, we used full-thickness excisional wounds in PM mice to examine the extent of wound closure, the level of neutrophil infiltration and the temporal expression levels of inhibitory factor kappa B alpha (IκBα), IL-1β and TNF-α mRNA at the wound site in the CuZnSOD overexpresser and knockout mice, as well as in the NAC-supplemented mice during the early stage of wound healing. In these experiments, we used ad libitum feeding with a very low protein diet, a common experimental model of PM that produces severe malnutrition, in order to gain further insight into the pathobiology of this perplexing clinical condition.

2. Methods and materials

2.1. Animals and diets

Female CD-1 mice (4–6 weeks old, 21–25 g) bred in our laboratory were used in all experiments. For experiments involving CuZnSOD transgenic mice, CuZnSOD overexpresser (CuZnSOD+++), wild-type (CuZnSOD+/+) and CuZnSOD knockout (CuZnSOD−/−) mice were selected by genotype and pedigree analysis as previously detailed [25]. The CuZnSOD protein levels and enzyme activity of transgenic, wild-type and knockout mice were analyzed to demonstrate the variable CuZnSOD expressions in these mice as previously shown [25]. Mice were housed in individual cages in a temperature- and humidity-controlled room (12-h light/dark cycle) with free access to tap water and diet. Mice were acclimated for 2 days before initiation of dietary treatments. AIN-93G-purified rodent powder diet (Dyets, Bethlehem, PA) was formulated to be isocaloric and contained either 5 g/kg protein diet (PM) or 150 g/kg protein diet (control). The composition of the control and PM diet has been published previously [26]. The amount of NAC (Sigma, St. Louis, MO) added to the PM diet was calculated so that the supplemented diet (PM+NAC) had the same amount of sulfur-containing amino acids as the control diet.

Mice were fed either the PM or the control diet for 3 weeks. *N*-Acetylcysteine-supplemented animals received the PM diet for 2 weeks, followed by a 1-week treatment with PM+NAC diet. Wounds were made at the end of the third week. Mice with variable CuZnSOD expression (overexpressed, wild type and knockout) were fed a control or a PM diet for 3 weeks before cutaneous wounds were made to test if CuZnSOD expression plays a role during the early stage of wound healing. All mice were used in accordance with animal protocols approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee.

2.2. Wound biopsy

Mice were anaesthetized with isoflurane, and the back of the mouse was shaved and sterilized using an alcohol swab. The wound biopsy model used in this experiment was described previously [4]. The shaven skin was then pinched and folded, and a sterile biopsy punch (3.5-mm-diameter, Miltex Instrument, York, PA) was used to punch through the full thickness of the folded skin. This yielded two circular wounds identical in size on the dorsum, below the shoulder blades, of each mouse. A wound placed in this area cannot be reached by the mice and, therefore, prevents self-licking.

2.3. Measurement of wound closure

Measurement of wound closure was described previously [27]. Wounds from individual mice were digitally photographed every day, beginning on the day of wounding (day 0). A standard "dot," equivalent in size to the initial wound area, was placed beside the wound and used as a reference. Wound size was then calculated by determining the area of the wound in comparison to the area of the standard dot. For all measurements, wound size was quantified using Canvas 7SE software (Deneba, Miami, FL). Wound closure was expressed as the ratio of wound area (each day after wounding). A smaller wound ratio indicates faster wound closure.

2.4. Harvesting and histological analysis

Harvesting and histological preparation of wound tissues were previously described [27]. Mice were euthanized with an overdose of isoflurane to collect tissue samples at the wound site for examination of neutrophil infiltration into the wound area. Wounds at 0, 6, 12 and 24 h after wounding were removed by cutting a square area that encompassed the entire wound site from four mice from each treatment group. Harvested tissues were immediately stored in 4% formaldehyde solution in phosphate-buttered saline (PBS,

pH 7.4) and were then washed in PBS, dehydrated in series of alcohols and embedded in paraffin. Microtome sections (5-μm-thick) were cut vertically across the wound site, adhered to the slides and were stained with hematoxylin and eosin. Photographs of the wound site were obtained, and the images were digitized using Adobe Photoshop (Adobe Systems, Mountain View, CA). Neutrophil infiltration was quantified by densitometric analysis of the wound site using ImageJ (NIH, Bethesda, MD).

2.5. In situ hybridization (immunofluorescence)

The in situ hybridization protocols were performed as described previously for ribonucleotide (cDNA) probes [4,26,28]. Antisense probes were transcribed using the Riboprobe System (Promega Biotech, Madison, WI) with T7 RNA polymerase. Mouse cDNAs of IκBα and TNF-α were generously provided by Dr. Rebecca Taub (University of Pennsylvania, Philadelphia, PA) and Dr. Karl Decker (Albert-Ludwigs-Universitat, Freiburg, Germany), respectively. A 672-bp DNA sequence of murine IL-1β mRNA was prepared from commercially available insert #963357 in vector pT7T3D-pac cloned in the host *Escherichia coli* (American Type Culture Collection, Manassas, VA). Immunofluorescence kits were obtained from Dako (Carpinteria, CA). The mRNA production was quantified by measuring the fluorescence intensity of cells expressing the mRNA of IκBα, IL-1β and TNF-α using an ImageJ software.

2.6. Statistical analysis

Data are reported as means±S.E.M. Statistical comparisons of the effect of diet on wound size were determined at each time point using the two-tailed Student *t* test (Fig. 1) or one-way analysis of variance (Fig. 2), followed by Duncan's new multiple range test applied at the 5% level of significance. For all other figures, the data were analyzed by the general linear model procedure to determine significant main effects, and by the least significant mean test to

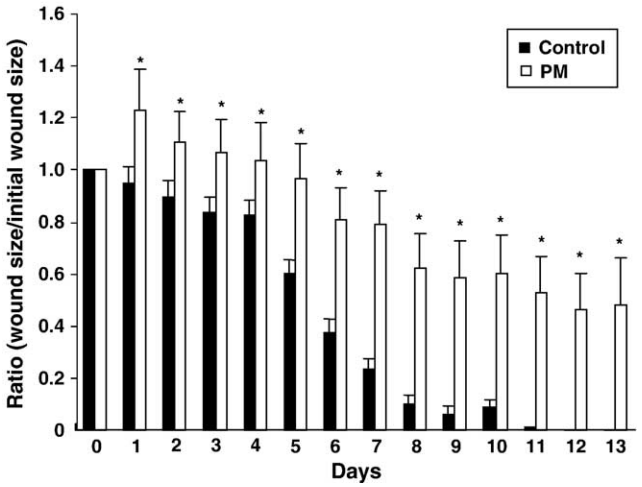

Fig. 1. Wound closure during cutaneous wound healing in mice fed a control (150 g/kg protein) or a PM (5 g/kg protein) diet for 3 weeks. The area of the wound of any time point was relative to the area of the wound on day 0 (set at 1.0). Values are mean±S.E.M., $n=6$. Values marked with an asterisk are significantly different ($P<.05$) from the control group at the same time point.

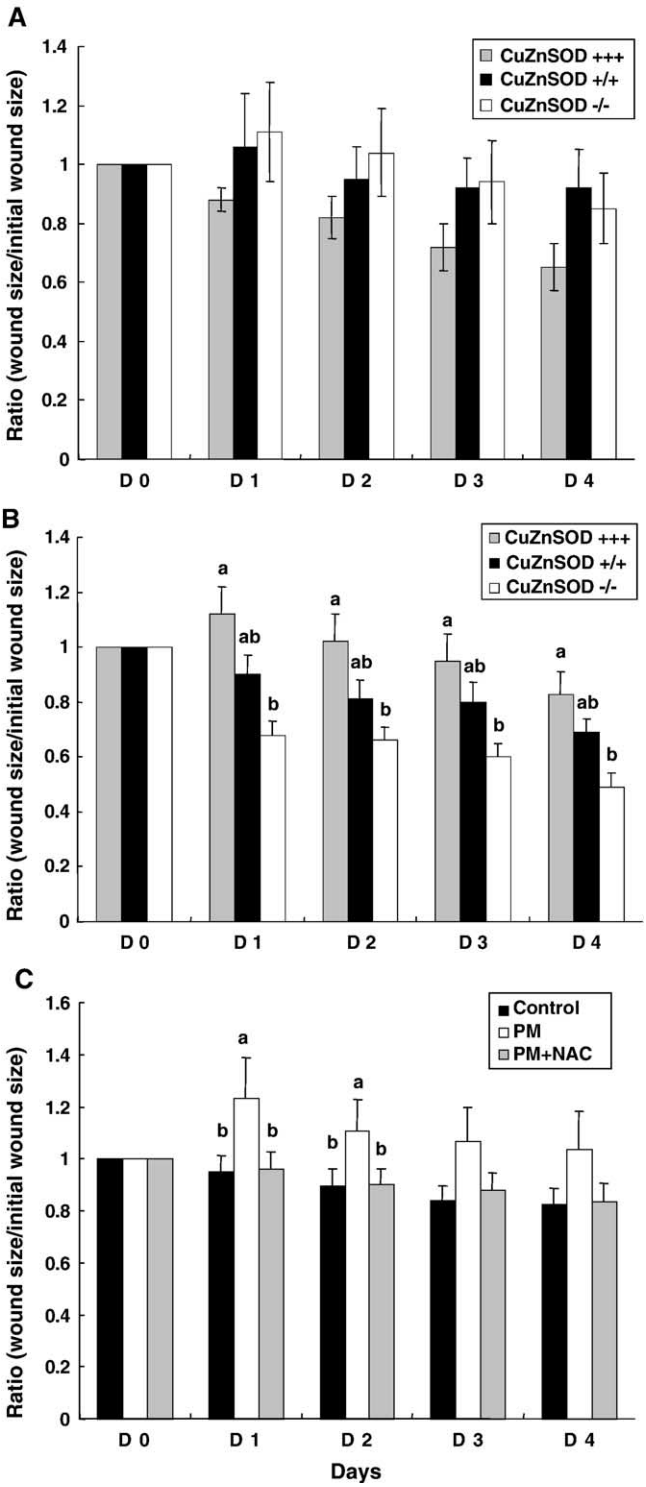

Fig. 2. Wound closure during early cutaneous wound healing in mice with variable CuZnSOD gene expression fed a PM (A) or a control (B) diet for 3 weeks and in wild-type mice fed a PM, a control diet for 3 weeks or a PM diet supplemented with NAC for the final week (C). The area of the wound of any time point was relative to the area of the wound on day 0 (set at 1.0). Values are mean±S.E.M., $n=6$. Means at a time without a common letter differ, $P<.05$.

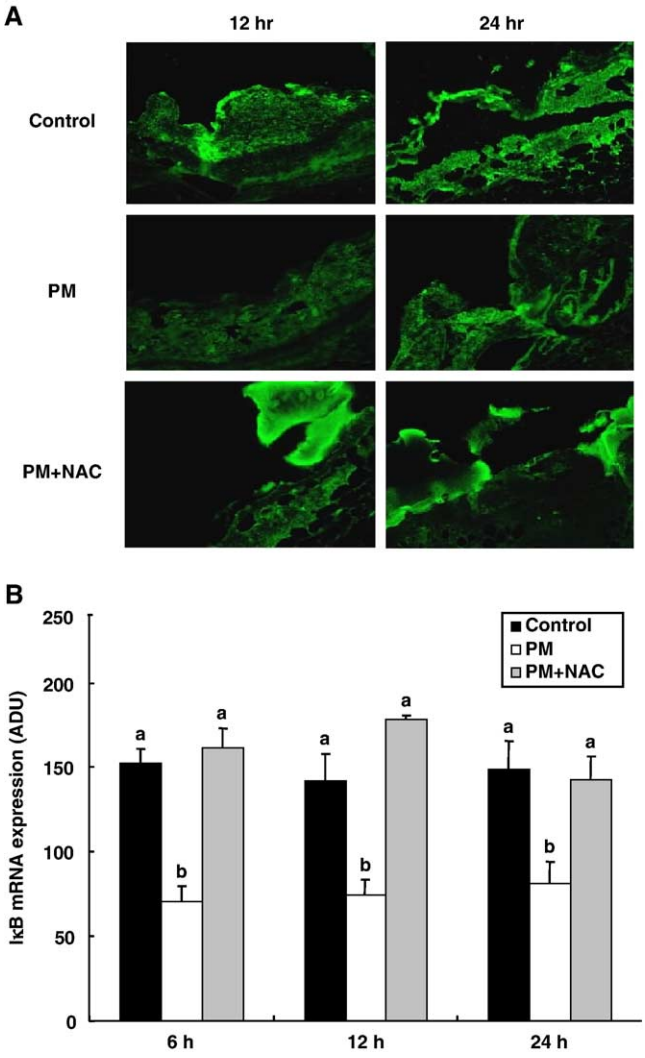

Fig. 3. Inhibitory factor kappa B alpha mRNA expression in cutaneous wounds of mice fed a PM, control or PM+NAC diet for 3 weeks. (A) Representative photographs of in situ hybridization fluorescence with a digoxigenin-labeled antigen probe. Top row, 12 and 24 h after wounding in control mice. Middle row, 12 and 24 h after wounding in PM mice. Bottom row, 12 and 24 h after wounding in PM mice with NAC supplementation. (B) Quantified IκBα mRNA expression determined by densitometry. Values are mean±S.E.M., $n=4$. Means at a time without a common letter differ, $P<.05$. ADU indicates arbitrary density unit.

determine significant differences between means. Results were considered significant at $P<.05$.

3. Results

3.1. Protein malnutrition model

The model of PM was successfully established by feeding a very low protein diet containing 5 g/kg protein (PM diet). Food consumption (gram diet per day) did not significantly differ between animals fed the control diet, the PM diet or the PM diet supplemented with NAC. However, mice fed either the PM or the PM+NAC diet exhibited a drastic reduction in body weight from 20.6±0.4 (mean±S.E.M.) to 15±0.3 g, whereas mice fed a control diet exhibited an increase from 21.1±0.5 to 25.7±0.4 g in body weight. Similar results of food consumption and body weight gain and loss were obtained in the study in which CuZnSOD+++, CuZnSOD+/+ and CuZnSOD−/− were fed a control or PM diet. In addition to the body weight loss, light microscopic examination of liver tissue demonstrated a dramatic nuclear shrinkage, a hallmark feature of apoptosis in the hepatocytes of PM mice (data not shown). In addition to nuclear shrinkage, the cytoplasmic content of hepatocytes was markedly decreased in PM mice compared to control, results consistent with extensive fat degeneration in PM animals.

3.2. Protein malnutrition and wound closure

To examine the effect of PM on wound closure during cutaneous wound healing, we determined the wound size from day 0 until completely healed in nontransgenic mice fed

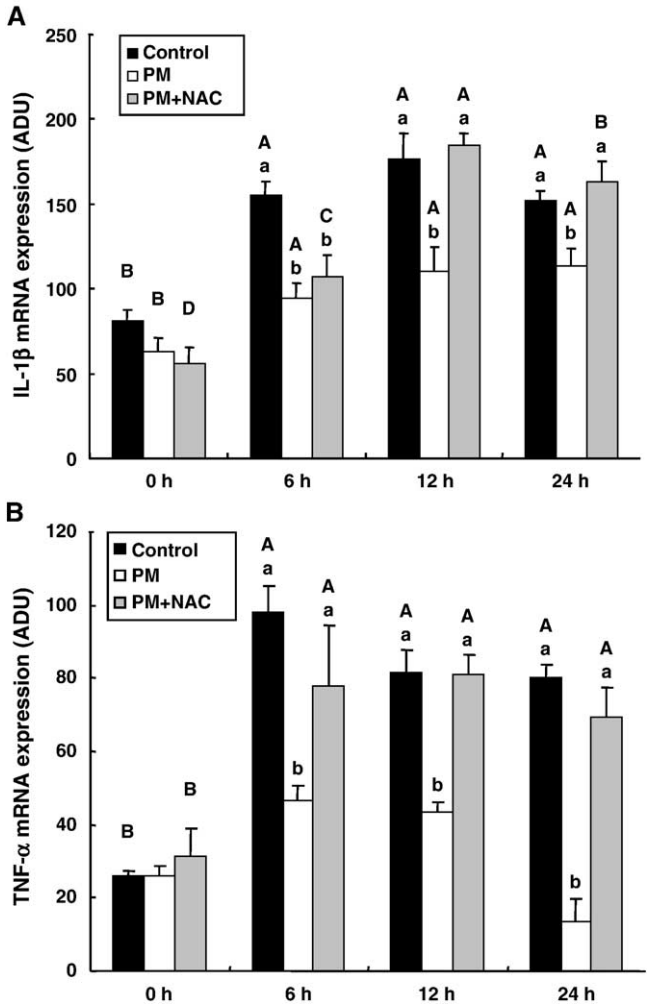

Fig. 4. Interleukin-1β (A) and TNF-α (B) mRNA levels in cutaneous wounds of mice fed a PM, control or PM+NAC diet for 3 weeks. Interleukin-1β and TNF-α mRNA were determined by in situ hybridization fluorescence and quantified by densitometry. Values are mean±S.E.M., $n=4$. Means at a time without a common letter differ, $P<.05$. ADU indicates arbitrary density unit.

a PM or a control diet. As illustrated in Fig. 1, wound size increased significantly at day 1 and remained significantly larger throughout the wound healing period in mice fed the PM diet compared to mice fed the control diet.

3.3. Reactive oxygen species, NAC supplementation and wound closure

To assess the influence of ROS on wound healing in PM during the early inflammatory stage, we determined the wound size from day 0 until day 4 of the healing process in CuZnSOD+++, CuZnSOD+/+ and CuZnSOD−/− transgenic mice fed a control or PM diet. As illustrated in Fig. 2A, there was a trend toward an enhancement of wound closure in CuZnSOD overexpresser mice compared to wide type and knockout mice fed a PM diet. In contrast, CuZnSOD overexpression had a dramatic detrimental effect on wound healing in mice fed a control diet. Wound size increased on day 1 in CuZnSOD+++ mice, whereas in CuZnSOD−/− mice, wound size decreased at the same time point (Fig. 2B). Furthermore, wound size was significantly greater in CuZnSOD+++ compared to CuZnSOD−/− mice throughout the 4-day study period ($P<.05$).

Based on these results, we next examined the effect of dietary supplementation with the antioxidant NAC on

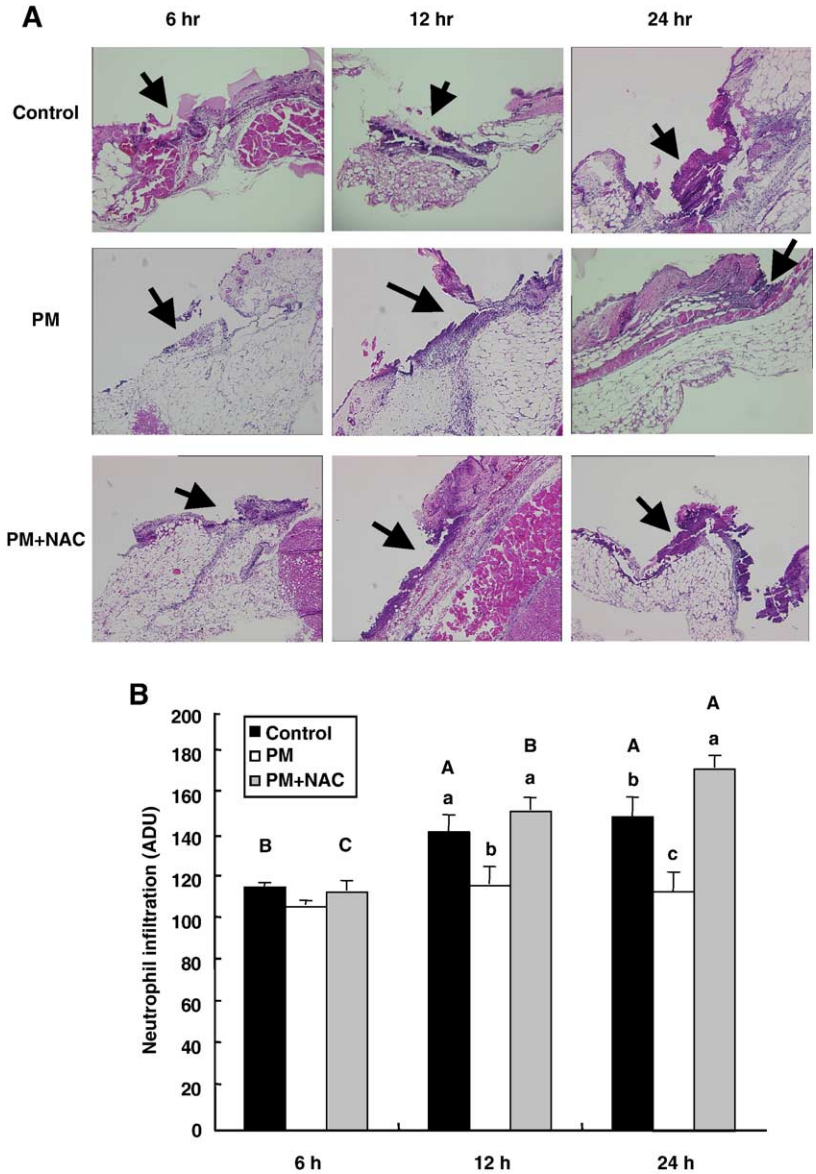

Fig. 5. Neutrophil infiltration into cutaneous wounds of mice fed a PM, control or PM+NAC diet for 3 weeks. (A) Representative photograph of neutrophil infiltration (arrow heads) into cutaneous wounds determined in hematoxylin and eosin stained sections. Top row, 6, 12 and 24 h after wounding in control mice. Middle row, 6, 12 and 24 h after wounding in PM mice. Bottom row, 6, 12 and 24 h after wounding in PM mice with NAC supplementation. (B) Densitometric analysis of neutrophil infiltration into cutaneous wounds. Values are expressed as mean±S.E.M., $n=4$. Means at a time without a common letter differ, $P<.05$. ADU indicates arbitrary density unit.

cutaneous wound healing in PM mice during the early inflammatory stage. As shown in Fig. 2C, wound closure was dramatically delayed in PM mice during the early inflammatory stage. Moreover, wound size was significantly greater in PM mice compared to controls and PM+NAC throughout the 4-day period ($P<.05$).

3.4. Protein malnutrition and the early inflammatory responses

The mRNA expression level of the proinflammatory cytokines IL-1β and TNF-α, as well as the expression of IκBα, a gene that is both regulated by and mirrors the expression of NFκB, was measured in an effort to characterize the molecular mechanisms that underlie impaired wound healing in PM. As demonstrated in Fig. 3, IκBα mRNA was detected at the wound site in all dietary treatments, but the expression level was significantly attenuated in PM mice when compared to control and PM+NAC mice. Notably, NAC supplementation restored IκBα mRNA levels in PM mice to that observed in control mice.

The temporal expression patterns of IL-1β and TNF-α mRNA are presented in Fig. 4. The expression of IL-1β mRNA in dermal tissue at the wound edge peaked at 12 h in both control and PM+NAC mice (Fig. 4A), whereas peak IL-1β expression was not observed in PM mice until 24 h. Moreover, the peak expression level of IL-1β in PM mice was significantly less than the peak IL-1β expression in either the control or the PM+NAC mice. Notably, peak expression of TNF-α was observed at 6 h in all treatment groups (Fig. 4B). However, the level of expression was significantly lower in PM mice compared to control or PM+NAC mice.

Histological analysis revealed that neutrophil infiltration was significantly depressed in PM mice compared to control and PM+NAC mice at 12 and 24 h (Fig. 5A, B). Thus, PM leads to a quantitative reduction in the level of neutrophil infiltration into the wound site up to 24 h after wounding. Moreover, the level of neutrophil infiltration was restored to control levels in PM mice supplemented with NAC. Taken together, these findings demonstrated that the early inflammatory response is dramatically attenuated in PM mice, and that supplementation of PM mice with NAC can effectively restore features of the early inflammatory response to levels observed in control mice.

4. Discussion

In this study, we have demonstrated in a cutaneous wound healing model that wound closure is significantly prolonged in PM mice (Fig. 1). In addition to a lack of appropriate substrates, particularly essential amino acids that may impair the latter stages of wound healing such as tissue repair and remodeling in PM, the adverse effects of PM may arise during the early inflammatory phase of wound healing as a result of excessive ROS generation. In our experiments, wound closure was significantly delayed, and specific markers of the early inflammatory response, namely, the mRNA expression levels of IκBα, IL-1β and TNF-α, and the neutrophil infiltration, were reduced in PM mice. Moreover, our data clearly demonstrate that NAC supplementation of PM animals enhanced wound closure and restored markers of the inflammatory response to levels similar to control animals. These findings corroborate our hypothesis that delayed wound healing in PM occurs as a consequence of excessive ROS generation and the subsequent deregulation of signaling molecules that are critical mediators of the inflammatory response.

Our data also demonstrated that ROS may play the role of a double-edged sword. We used mice with variable CuZnSOD genotypes to test the function of ROS in wound healing process. CuZnSOD functions as an antioxidant by catalyzing the dismutation of $O_2^{\cdot-}$ [29]. Overexpression of CuZnSOD has been shown to reduce cellular ROS burden by lowering the steady-state levels of both $O_2^{\cdot-}$ and H_2O_2 [30,31]. Thus, our observations that wound closure was enhanced in CuZnSOD+++ mice with PM (Fig. 2A) are consistent with the notion that impaired wound healing in PM may be attributable to excessive ROS production. In contrast, we also observed an enhanced wound closure in CuZnSOD−/− mice fed a control diet compared to CuZnSOD+++ mice (Fig. 2B). This apparent paradox may indicate that although excessive ROS production can impede wound healing, an unspecified quantity of ROS is nevertheless essential to the wound healing process. Recently, clinical treatments using hyperbaric oxygen therapy have demonstrated that increased oxygen tension at the wound site increases the formation of granulation tissue and accelerates the wound closure [32,33]. Although hyperoxia has been shown to increase ROS generation [34], the physiological bases for hyperoxia-mediated improved wound healing remain largely unknown.

If excessive ROS generation impairs wound healing in PM, then agents that counteract the effects of ROS should improve wound healing. Indeed, we have clearly demonstrated that dietary supplementation with NAC enhanced wound closure during the early inflammatory stage in PM mice (Fig. 2C). The ability of NAC to improve wound healing may be attributable to its role as an antioxidant. In fact, there are several mechanisms by which NAC may function as an antioxidant. First, NAC has been shown to react directly with various ROS, including H_2O_2, $O_2^{\cdot-}$ and the hydroxyl radical $^{\cdot}OH$ [35]. Second, NAC is a cysteine prodrug and may exert its antioxidant effects by enhancing tissue levels of GSH [26]. Glutathione is a cysteine-containing tripeptide that functions as a substrate in numerous physiological reaction pathways, including the GSH peroxidase-catalyzed reduction of organic peroxides as well as the GSH transferase-catalyzed detoxification of xenobiotics [36]. Hence, the antioxidant properties of NAC may enhance wound healing in PM mice either directly via its detoxification of ROS or indirectly by facilitating GSH biosynthesis. Notably, numerous reports have demonstrated

that tissue injury is coupled with GSH depletion [37–39] and that GSH depletion is a hallmark feature of PM [26,40,41].

What is the mechanism of enhanced wound healing in PM mice supplemented with NAC? We addressed this question by examining the activity of NFκB through the expression of IκBα, an immediate early gene whose expression mirrors the activity of NFκB [42] (Fig. 3). Nuclear factor kappa B is a redox-sensitive transcription factor that plays a central role in inflammation by responding to and amplifying numerous inflammatory pathways. It has been widely demonstrated that ROS can directly activate NFκB, whereas the application of antioxidants or the up-regulation of antioxidants can prevent NFκB activity [43–46]. However, the regulation of NFκB activity by ROS is very complex, and there is an evidence that extremely high levels of oxidative stress can in fact lead to the inhibition of NFκB rather than to its activation [47]. We therefore reasoned that because antioxidant defenses are depressed in PM [48–51], dietary supplementation with the antioxidant NAC may lessen ROS-mediated inactivation of NFκB and improve immune function. Indeed, the data presented herein unequivocally demonstrate that supplementation of the antioxidant NAC up-regulates NFκB activity in PM and that this coincides with improved wound healing.

To further confirm the effect of NAC on NFκB activation and the events surrounding the inflammatory response during wound healing, we examined the mRNA expression of the proinflammatory cytokines IL-1β and TNF-α, which are regulated by NFκB [8,52]. Our experiments demonstrated that the mRNA expression of IL-1β and TNF-α at the wound site was reduced in PM mice at each time point, and that NAC supplementation to PM mice restored IL-1β and TNF-α expression to normal levels (Fig. 4A, B). Interestingly, the expression of IL-1β and TNF-α precedes the expression of several growth factors vital to the wound repair and remodeling process, including vascular endothelial growth factor [53] and keratinocyte growth factor [54]. Thus, the appropriate expression level of IL-1β and TNF-α may be of major significance for the induction of growth factors during wound healing.

In addition to their putative role in stimulating the expression of growth factor genes that are important in cutaneous wound healing [55], IL-1β and TNF-α modulate the expression of chemokines and adhesion molecules necessary for the recruitment of inflammatory cells to the site of injury [56,57]. Although we did not address this directly, we did observe a significant decrease in neutrophil infiltration into the wound site of PM mice compared to controls and, additionally, that neutrophil infiltration was elevated to control values in PM mice supplemented with NAC (Fig. 5). These findings further suggest a negative role for ROS by inhibiting neutrophil infiltration during the early inflammatory response in PM mice. Neutrophils are the first inflammatory cells to infiltrate the wound site and play a key role during the early inflammatory stage of wound healing by clearing the site of contaminating bacteria and guarding against infection. Moreover, recent observations have shown that neutrophils are major producers of numerous cytokines that serve as some of the earliest signals to activate keratinocytes and fibroblasts, and, thus, initiate the proliferative and remodeling phases of wound healing [3,58].

Taken together, the results of this work support the hypothesis that excessive ROS generation contributes to the delayed wound healing and immunodepression observed in PM. Furthermore, our findings provide a model explanation for the deleterious effects of PM on wound healing during the early inflammatory stage. It can be envisioned that in PM, excessive ROS formation inhibits NFκB activation either through the oxidative modification of critical DNA-binding residues within NFκB or through oxidative inactivation of key regulatory enzymes that regulate NFκB activity. Regardless, because NFκB occupies a central role in the inflammatory process and is required for the induction of proinflammatory cytokines such as IL-1β and TNF-α, ROS-mediated inactivation of NFκB may lead to a catastrophic inhibition of the inflammatory cascade, culminating in the immunosuppression and delayed wound healing that marks the morbidity and mortality of PM.

In addition, this work has demonstrated that dietary supplementation of NAC during the early stage of wound healing can correct the impaired wound healing and delayed inflammatory response observed in PM mice. Indeed, it is remarkable that a brief period of dietary NAC supplementation totally abrogates the delayed wound healing and immunodepression observed in PM. *N*-Acetylcysteine is well tolerated in humans and has been used as an adjuvant therapy in clinical practice. However, this is the first study to show that NAC supplementation may be an effective therapeutic strategy to enhance wound healing during the early rehabilitation of PM patients.

In summary, the current study demonstrated that ROS exert negative effects on the early inflammatory response of cutaneous wound healing in a mouse model of PM. Understanding the molecular mechanisms that regulate cutaneous wound healing is critical not only in PM, but also in numerous other disorders associated with abnormal wound repair. Hence, the results of this work may provide critical insight into future nutritional intervention strategies designed to enhance immune function not only in patients suffering from PM, but also in patients suffering from malnutrition-related disorders.

References

[1] Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev 2003;83:835–70.
[2] Abbott RE, Corral CJ, MacIvor DM, Lin X, Ley TJ, Mustoe TA. Augmented inflammatory responses and altered wound healing in cathepsin G-deficient mice. Arch Surg 1998;133:1002–6.
[3] Hubner G, Brauchle M, Smola H, Madlener M, Fassler R, Werner S. Differential regulation of pro-inflammatory cytokines during wound

healing in normal and glucocorticoid-treated mice. Cytokine 1996;8: 548–56.
[4] Mercado AM, Quan N, Padgett DA, Sheridan JF, Marucha PT. Restraint stress alters the expression of interleukin-1 and keratinocyte growth factor at the wound site: an in situ hybridization study. J Neuroimmunol 2002;129:74–83.
[5] Mercado AM, Padgett DA, Sheridan JF, Marucha PT. Altered kinetics of IL-1 alpha, IL-1 beta, and KGF-1 gene expression in early wounds of restrained mice. Brain Behav Immun 2002;16:150–62.
[6] Delgado AV, McManus AT, Chambers JP. Production of tumor necrosis factor-alpha, interleukin 1-beta, interleukin 2, and interleukin 6 by rat leukocyte subpopulations after exposure to substance P. Neuropeptides 2003;37:355–61.
[7] Gillitzer R, Goebeler M. Chemokines in cutaneous wound healing. J Leukoc Biol 2001;69:513–21.
[8] Bowie A, O'Neill LA. Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol 2000;59:13–23.
[9] Rhodus NL, Cheng B, Myers S, Bowles W, Ho V, Ondrey F. A comparison of the pro-inflammatory, NF-kappaB-dependent cytokines: TNF-alpha, IL-1-alpha, IL-6, and IL-8 in different oral fluids from oral lichen planus patients. Clin Immunol 2005;114:278–83.
[10] Barbul A. Immune aspects of wound repair. Clin Plast Surg 1990;17: 433–42.
[11] Takahashi S, Fujita T, Yamamoto A. Role of nuclear factor-kappaB in gastric ulcer healing in rats. Am J Physiol Gastrointest Liver Physiol 2001;280:G1296–304.
[12] Haas AF, Wong JW, Iwahashi CK, Halliwell B, Cross CE, Davis PA. Redox regulation of wound healing? NF-kappaB activation in cultured human keratinocytes upon wounding and the effect of low energy HeNe irradiation. Free Radic Biol Med 1998;25: 998–1005.
[13] Scevola D, Di Matteo A, Uberti F, Minoia G, Poletti F, Faga A. Reversal of cachexia in patients treated with potent antiretroviral therapy. AIDS Read 2000;10:365–9, 71–5.
[14] Ambrus Sr JL, Ambrus Jr JL. Nutrition and infectious diseases in developing countries and problems of acquired immunodeficiency syndrome. Exp Biol Med (Maywood) 2004;229:464–72.
[15] Okita M. Chronic hepatic disease and dietary instruction. Hepatol Res 2004;30S:92–5.
[16] Thomas DR. The role of nutrition in prevention and healing of pressure ulcers. Clin Geriatr Med 1997;13:497–511.
[17] Zaizen Y, Ford EG, Costin G, Atkinson JB. Stimulation of wound bursting strength during protein malnutrition. J Surg Res 1990;49: 333–6.
[18] Chandra RK. Protein-energy malnutrition and immunological responses. J Nutr 1992;122:597–600.
[19] Chandra RK. Nutrition and the immune system: an introduction. Am J Clin Nutr 1997;66:460S–3S.
[20] Ashour MN, Salem SI, El-Gadban HM, Elwan NM, Basu TK. Antioxidant status in children with protein-energy malnutrition (PEM) living in Cairo, Egypt. Eur J Clin Nutr 1999;53:669–73.
[21] Doherty JF, Golden MH, Remick DG, Griffin GE. Production of interleukin-6 and tumour necrosis factor-alpha in vitro is reduced in whole blood of severely malnourished children. Clin Sci (Lond) 1994; 86:347–51.
[22] Munoz C, Arevalo M, Lopez M. Impaired interleukin-1 and tumor necrosis factor production in protein-calorie malnutrition. Nutr Res 1994;14:347–52.
[23] Sauerwein RW, Mulder JA, Mulder L, Lowe B, Peshu N, Demacker PN, et al. Inflammatory mediators in children with protein-energy malnutrition. Am J Clin Nutr 1997;65:1534–9.
[24] Lotfy OA, Saleh WA, el-Barbari M. A study of some changes of cell-mediated immunity in protein energy malnutrition. J Egypt Soc Parasitol 1998;28:413–28.
[25] Levy MA, Tsai YH, Reaume A, Bray TM. Cellular response of antioxidant metalloproteins in Cu/Zn SOD transgenic mice exposed to hyperoxia. Am J Physiol Lung Cell Mol Physiol 2001;281: L172–82.
[26] Li J, Quan N, Bray T. Supplementation of *N*-acetylcysteine normalizes lipopolysaccharide-induced nuclear factor B activation and proinflammatory cytokine production during early rehabilitation of protein malnourished mice. J Nutr 2002;132:3286–92.
[27] Lim Y, Levy M, Bray TM. Dietary zinc alters early inflammatory responses during cutaneous wound healing in weanling CD-1 mice. J Nutr 2004;134:811–6.
[28] Whitfield Jr HJ, Brady LS, Smith MA, Mamalaki E, Fox RJ, Herkenham M. Optimization of cRNA probe in situ hybridization methodology for localization of glucocorticoid receptor mRNA in rat brain: a detailed protocol. Cell Mol Neurobiol 1990;10:145–57.
[29] Fridovich I. Superoxide radical and superoxide dismutases. Annu Rev Biochem 1995;64:97–112.
[30] Liochev SI, Fridovich I. The role of O_2^- in the production of $HO^{\cdot}$: in vitro and in vivo. Free Radic Biol Med 1994;16:29–33.
[31] Teixeira HD, Schumacher RI, Meneghini R. Lower intracellular hydrogen peroxide levels in cells overexpressing CuZn-superoxide dismutase. Proc Natl Acad Sci U S A 1998;95:7872–5.
[32] Boykin JJ. The nitric oxide connection: hyperbaric oxygen therapy, becaplermin, and diabetic ulcer management. Adv Skin Wound Care 2000;13(4 Pt 1):169–74.
[33] Williams RL. Hyperbaric oxygen therapy and the diabetic foot. J Am Podiatr Med Assoc 1997;87:279–92.
[34] Freeman BA, Crapo JD. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. J Biol Chem 1981;256: 10986–92.
[35] Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of *N*-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med 1989;6:593–7.
[36] Balendiran GK, Dabur R, Fraser D. The role of glutathione in cancer. Cell Biochem Funct 2004;22:343–52.
[37] Shukla A, Rasik AM, Patnaik GK. Depletion of reduced glutathione, ascorbic acid, vitamin E and antioxidant defence enzymes in a healing cutaneous wound. Free Radic Res 1997;26:93–101.
[38] Nagasaki H, Nakano H, Boudjema K, Jaeck D, Alexandre E, Baek Y, et al. Efficacy of preconditioning with *N*-acetylcysteine against reperfusion injury after prolonged cold ischaemia in rats liver in which glutathione had been reduced by buthionine sulphoximine. Eur J Surg 1998;164:139–46.
[39] Ding HQ, Zhou BJ, Liu L, Cheng S. Oxidative stress and metallothionein expression in the liver of rats with severe thermal injury. Burns 2002;28:215–21.
[40] Meister A, Anderson ME. Glutathione. Annu Rev Biochem 1983;52: 711–60.
[41] Oumi M, Miyoshi M, Yamamoto T. Ultrastructural changes and glutathione depletion in the skeletal muscle induced by protein malnutrition. Ultrastruct Pathol 2001;25:431–6.
[42] Miyamoto S, Verma IM. Rel/NF-kappa B/I kappa B story. Adv Cancer Res 1995;66:255–92.
[43] Renard P, Zachary MD, Bougelet C, Mirault ME, Haegeman G, Remacle J, et al. Effects of antioxidant enzyme modulations on interleukin-1-induced nuclear factor kappa B activation. Biochem Pharmacol 1997;53:149–60.
[44] Sen CK, Packer L. Antioxidant and redox regulation of gene transcription. Faseb J 1996;10:709–20.
[45] Son EW, Mo SJ, Rhee DK, Pyo S. Vitamin C blocks TNF-alpha-induced NF-kappaB activation and ICAM-1 expression in human neuroblastoma cells. Arch Pharm Res 2004;27:1073–9.
[46] Prasad AS, Bao B, Beck FW, Kucuk O, Sarkar FH. Antioxidant effect of zinc in humans. Free Radic Biol Med 2004;37:1182–90.
[47] Marshall HE, Merchant K, Stamler JS. Nitrosation and oxidation in the regulation of gene expression. Faseb J 2000;14:1889–900.
[48] Golden MH, Ramdath D. Free radicals in the pathogenesis of kwashiorkor. Proc Nutr Soc 1987;46:53–68.

[49] Sive AA, Subotzky EF, Malan H, Dempster WS, Heese HD. Red blood cell antioxidant enzyme concentrations in kwashiorkor and marasmus. Ann Trop Paediatr 1993;13:33–8.
[50] Becker K, Botticher D, Leichsenring M. Antioxidant vitamins in malnourished Nigerian children. Int J Vitam Nutr Res 1994;64: 306–10.
[51] Manary MJ, Leeuwenburgh C, Heinecke JW. Increased oxidative stress in kwashiorkor. J Pediatr 2000;137:421–4.
[52] Blackwell TS, Christman JW. The role of nuclear factor-kappa B in cytokine gene regulation. Am J Respir Cell Mol Biol 1997; 17:3–9.
[53] Frank S, Hubner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem 1995;270:12607–13.
[54] Werner S, Peters KG, Longaker MT, Fuller-Pace F, Banda MJ, Williams LT. Large induction of keratinocyte growth factor expression in the dermis during wound healing. Proc Natl Acad Sci U S A 1992;89: 6896–900.
[55] Brauchle M, Fassler R, Werner S. Suppression of keratinocyte growth factor expression by glucocorticoids in vitro and during wound healing. J Invest Dermatol 1995;105:579–84.
[56] Bickel M, Nothen SM, Freiburghaus K, Shire D. Chemokine expression in human oral keratinocyte cell lines and keratinized mucosa. J Dent Res 1996;75:1827–34.
[57] Zhang X, Kohli M, Zhou Q, Graves DT, Amar S. Short- and long-term effects of IL-1 and TNF antagonists on periodontal wound healing. J Immunol 2004;173:3514–23.
[58] Nian M, Lee P, Khaper N, Liu P. Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res 2004;94:1543–53.