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Myosin phosphatase accelerates cutaneous wound healing by regulating migration and differentiation of epidermal keratinocytes via Akt signaling pathway in human and murine skin

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ABSTRACT

Wound healing is a complex sequence of cellular and molecular processes such as inflammation, cell migration, proliferation and differentiation. ROCK is a widely investigated Ser/Thr kinase with important roles in re-arranging the actomyosin cytoskeleton. ROCK inhibitors have already been approved to improve corneal endothelial wound healing. The purpose of this study was to investigate the functions of myosin phosphatase (MP or PPP1CB), a type-1 phospho-Ser/Thr-specific protein phosphatase (PP1), one of the counter enzymes of ROCK, in skin homeostasis and wound healing. To confirm our hypotheses, we applied tautomycin (TM), a selective PP1 inhibitor, on murine skin that caused the arrest of wound closure. TM suppressed scratch closure of HaCaT human keratinocytes without having influence on the survival of the cells. Silencing of, the regulatory subunit of MP (MYPT1 or PPP1R12A), had a negative impact on the migration of keratinocytes and it influenced the cell-cell adhesion properties by decreasing the impedance of HaCaT cells. We assume that MP differentially activates migration and differentiation of keratinocytes and plays a key role in the downregulation of transglutaminase-1 in lower layers of skin where no differentiation is required. MAPK Proteome Profiler analysis on human *ex vivo* biopsies with MYPT1-silencing indicated that MP contributes to the mediation of wound healing by regulating the Akt signaling pathway. Our findings suggest that MP plays a role in the maintenance of normal homeostasis of skin and the process of wound healing.

1. Introduction

Wound healing is a complex sequence of cellular and molecular processes consisting of hemostasis, inflammation, new tissue formation, re-epithelialization, and tissue remodeling [1–3]. Normal wound healing in skin is essential to reduce complications, including bacterial infection, water loss, and scar formation, that might perturb organ function and cause increased global morbidity and mortality [4,5]. Understanding the molecular, cellular, and physiologic aspects of wound healing is essential for developing new strategies to overcome impaired wound healing.

The epidermis consists of multiple layers, and cross-talk between the proliferation, migration, and differentiation processes is required for reepithelialization during wound healing [6]. Keratinocytes of the basal layer (*stratum basale*) adhere to the basement membrane and proliferate. Keratinocytes proliferate and migrate upwards through the granular and spinous layers [7], and eventually reach the cornified, outermost layers to replace cells lost through terminal differentiation [1]. In wounded skin, the keratinocytes near the damage zone respond to an array of signals, including growth factors, by activating both proliferation and migration, followed by differentiation [8]. A few days after wounding, monocytes appear in the wound and differentiate into

Abbreviations: PP1, protein phosphatase-1; PP1c, PP1 catalytic subunit; TM, tautomycin; H1152, (*S*)-(+)-2-Methyl-1-[(4-methyl-5-isoquinolinyl)sulfonyl]-hexahydro-1H-1,4-diazepine dihydrochloride; PBS, phosphate buffer saline; TBS, Tris-buffered saline solution; Tgase-1, transglutaminase-1; MP, myosin phosphatase; MYPT1, myosin phosphatase targeting subunit; NHEK, normal human keratinocyte

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macrophages [9], and new tissue formation follows. At this stage, keratinocytes migrate into the injured area for primary coverage and start to proliferate and differentiate to recover the stratification and to restore the barrier function of the epithelium. Moreover, fibroblasts transform to myofibroblasts, produce extracellular matrix, and contract the tissue [10]. Remodeling, the third stage of wound healing, starts 10 days–3 weeks after injury and can last up to a year. A majority of endothelial cells, macrophages, and myofibroblasts disappear by apoptosis, leaving behind the scar containing mostly collagen, a few cells, and extracellular matrix proteins.

Many steps in wound repair are poorly characterized at the molecular level, including the underlying mechanism of reepithelialization, a major step in new tissue formation. One of the most important events is the reversible phosphorylation of proteins catalyzed by protein kinases and phosphatases. The ligands of protein tyrosine kinase receptors (*e.g.* HGF, FGF, EGF) are positive regulators of reepithelialization, initiating the migration and proliferation of keratinocytes [5]. In contrast, migration of keratinocytes from the wound edge is enhanced by protein serine(Ser)/threonine(Thr) kinase inhibitors [11]. Acetylcholine acting through muscarinic receptors or catecholamines signaling *via* β2-adrenoreceptors, lead to activation of protein kinase A and G [12] or dephosphorylation of extracellular signal-related kinases (ERK1 and ERK2) by activation of protein phosphatase 2A [13]. Another Ser/Thr kinase involved in wound healing is Rho-associated protein kinase (ROCK). ROCK is the major downstream effector of small Rho GTPases, and is involved in various cellular functions that include actin cytoskeleton organization, cell-cell adhesion [14], cell motility, and anti-apoptotic processes [15]. ROCK plays a role in epithelial regeneration, and inhibition of ROCK significantly enhances cell proliferation, chemotaxis, and wound healing of periodontal ligament stem cells [16].

The role of protein kinases has been widely described in the process of wound healing but less is known about the protein phosphatases involved in these events. Okadaic acid, the inhibitor of protein phosphatase-1 (PP1) and -2A (PP2A) enzymes, increases the phosphorylation level of myosin II resulting in decreased hepatic cell migration [17]. Although enhanced myosin II phosphorylation is needed for migration, the inhibitory influence in this case is presumably due to a maintained phosphatase inhibition that would not allow “cycling” of myosin II phosphorylation required for effective migration. On the other hand, inhibition of PP2A by 10 nM okadaic acid resulted in an increased migration [13]. Changes in the phosphorylation level of Ca^{2+}-transport and contractile proteins upon phosphatase inhibition mediates cell migration and wound healing [18].

The cytoskeletal changes in the course of wound healing are correlated with the phosphorylation state of contractile proteins such as myosin II [19], ezrin/radixin/moesin, and adducin. Phosphorylation of these proteins is primarily regulated by ROCK [20] and its counter enzyme, a type 1 Ser/Thr specific protein phosphatase, termed myosin phosphatase (MP or PPP1CB). MP consists of a 38 kDa PP1 catalytic subunit (PP1c) of the β/δ isoform, a 130/133 kDa myosin phosphatase target subunit-1 (MYPT1 or PPP1R12A), and a 20 kDa subunit of unknown function in smooth muscle [21]. MP catalyzes the dephosphorylation of a variety of ROCK-phosphorylated proteins and may be a key player in re-epithelialization [22,23]. Therefore, we investigated the involvement of MP in the cellular processes and interactions required for proper regeneration of the skin. To address this hypothesis in a physiological setting, we used well-defined *in vivo* wound healing excision injury models of murine and human origins. We used a number of reconstituted *in vitro* cellular and *ex vivo* human skin models relevant to the wound healing processes to validate the observations in healing tissue.

Our present results suggest that the inhibition of PP1 suppresses wound healing without influencing cell survival. Silencing of MP has a negative impact on the migration of keratinocytes and also influences cell-cell adhesion properties by decreasing the impedance of the monolayer of MYPT1-silenced HaCaT cells. Here, we identify the signaling mechanism by which MP regulates wound healing through the modulation of Akt signaling and Hsp27. MP differentially activates migration and differentiation of keratinocytes and plays a key role in the downregulation of transglutaminase-1 (Tgase-1) in lower layers of skin where no differentiation is required.

2. Materials and methods

2.1. Materials

Tautomycin (Merck), H1152 (Santa Cruz Biotechnology), MYPT1 siRNA, Lipofectamine 2000 (ThermoFisher Scientific), protease inhibitor cocktail tablets (Roche), Texas Red-X phalloidin and ProLong Gold Antifade medium (Life Technologies) were from the indicated sources. All other chemicals were from Sigma-Aldrich unless otherwise indicated.

2.2. Antibodies

The following antibodies were obtained from the indicated sources or described in the respective references: Anti-MYPT1$^{1\text{-}296}$ [24] and anti-PP1cδ (Merck); anti-transglutaminase-1, anti-GAPDH, and anti-α-tubulin (Santa Cruz Biotechnology); anti-β-catenin (Abcam, Cambridge, UK); anti-Akt, anti-phospho-Akt Ser473, and horseradish peroxidase (HRP)-linked anti-mouse IgG (Cell Signaling Technology); HRP-conjugated anti-rabbit IgG (Sigma-Aldrich); Alexa Fluor 488 chicken anti-rabbit IgG (Life Technologies).

2.3. Ethics statement

All procedures were authorized by the Institutional Ethics Committee of University of Debrecen (protocol number 7/2010 DE MÁB v 9/2008/DEMÁB) and were conducted in accordance with the guidelines for the care and use of laboratory animals outlined in the national and European Union ethical regulations.

2.4. Wound healing study on mouse dorsal skin and tissue processing

Male Balb/c mice (Charles River) aged 6–7 week were housed individually in a controlled atmosphere in relative humidity of 35.0–75.0% with 12/12 h light and dark cycles and at a temperature range of 19.0–25.0 °C. Food and water were available *ad libitum*. Wound incision was performed as described previously [6]. The dorsal fur of the mice was depilated using commercial hair removal cream. Mice were anaesthetized with isoflurane inhalation (Abbott Animal Health, Abbott Park, IL, USA) prior to the surgical procedures and were kept warm during anesthesia. Three full thickness 4 mm punch biopsies were made on the dorsal surface of mice and the wound beds were wiped with povidone-iodine to prevent infection. The wounds arranged lengthways were made at the same time. Tautomycin (TM), a selective PP1 inhibitor, and H1152, a ROCK inhibitor, were applied on one of the wounds in hydrophilic anionic cream in a final concentration of 1 and 10 μM, respectively (n = 6 for each treatment). Control wounds were treated with hydrophilic cream only. Treatments were repeated daily for 6 days and wounds were covered with a surgical dressing to keep the cream in place. Animals were euthanized by cervical dislocation, then the skin was excised and the subcutaneous fat removed with a scalpel blade. The wound samples were transversely cut through exactly the center of the wound in each animal and were processed for histology, while the other halves were snap frozen in liquid nitrogen and stored at −80 °C for later analysis. Wound tissue samples were lysed with lysis buffer (25 mM Tris-HCl, pH 7.5, 0.6 M NaCl, 2 mM EDTA, 1 mM DTT, 0.5%(V/V) Triton X-100) containing protease inhibitor cocktail and phenylmethylsulfonyl fluoride (PMSF) using potter homogenizer as previously described [25]. Protein concentration was determined by