Journal of Dermatological Science 72 (2013) 32–44

Contents lists available at SciVerse ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Selective matrix (hyaluronan) interaction with CD44 and RhoGTPase signaling promotes keratinocyte functions and overcomes age-related epidermal dysfunction

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ARTICLE INFO

Article history:
Received 19 April 2013
Received in revised form 18 May 2013
Accepted 21 May 2013

Keywords:
Matrix hyaluronan
CD44
RhoGTPase signaling
Keratinocyte functions
Skin aging

ABSTRACT

Background: Mouse epidermal chronologic aging is closely associated with aberrant matrix (hyaluronan, HA)-size distribution/production and impaired keratinocyte proliferation/differentiation, leading to a marked thinning of the epidermis with functional consequence that causes a slower recovery of permeability barrier function.
Objective: The goal of this study is to demonstrate mechanism-based, corrective therapeutic strategies using topical applications of small HA (HA_S) and/or large HA (HA_L) [or a sequential small HA (HA_S) and large HA(HA_L) ($HA_s \rightarrow HA_L$) treatment] as well as RhoGTPase signaling perturbation agents to regulate HA/CD44-mediated signaling, thereby restoring normal epidermal function, and permeability barrier homeostasis in aged mouse skin.
Methods: A number of biochemical, cell biological/molecular, pharmacological and physiological approaches were used to investigate matrix HA-CD44-mediated RhoGTPase signaling in regulating epidermal functions and skin aging.
Results: In this study we demonstrated that topical application of small HA (HA_S) promotes keratinocyte proliferation and increases skin thickness, while it fails to upregulate keratinocyte differentiation or permeability barrier repair in aged mouse skin. In contrast, large HA (HA_L) induces only minimal changes in keratinocyte proliferation and skin thickness, but restores keratinocyte differentiation and improves permeability barrier function in aged epidermis. Since neither HA_S nor HA_L corrects these epidermal defects in aged CD44 knock-out mice, CD44 likely mediates HA-associated epidermal functions in aged mouse skin. Finally, blockade of Rho-kinase activity with Y27632 or protein kinase-Nγ activity with Ro31-8220 significantly decreased the HA (HA_S or HA_L)-mediated changes in epidermal function in aged mouse skin.
Conclusion: The results of our study show first that HA application of different sizes regulates epidermal proliferation, differentiation and barrier function in aged mouse skin. Second, manipulation of matrix (HA) interaction with CD44 and RhoGTPase signaling could provide further novel therapeutic approaches that could be targeted for the treatment of various aging-related skin disorders.

1. Introduction

Chronological skin aging is a universal and inevitable process characterized by physiological alterations in keratinocyte activities and epidermal function, as well as dermal changes independent of photo-induced alterations [1]. Epidermal dysfunction in aged skin can contribute to important clinical consequences, such as epidermal thinning (atrophy), permeability barrier dysfunction, xerosis/xerotic eczema, delayed wound healing, altered drug permeability, as well as increased susceptibility to ulceration and irritant contact dermatitis [1–3]. Yet, the cellular and molecular mechanisms that cause epidermal dysfunction during skin aging are not well understood, creating a strong rationale to elucidate alteration in the epidermal biology that underlies aged skin-related diseases.

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0923-1811/$36.00
http://dx.doi.org/10.1016/j.jdermsci.2013.05.003

In the epidermis, extracellular matrix (ECM) components, such as hyaluronan (HA), form an integral part of hemidesmosomes, and mediate keratinocyte attachment to the underlying basement membrane [4–6]. The dynamic nature of HA, particularly with regard to cellular interactions, is just beginning to be appreciated [4–6]. A general concept that has emerged from several studies is that HA fragments [Small Molecular Weight HA (HA_S)], and their larger precursor molecules [i.e., Large Molecular Weight HA (HA_L)] display distinct biological activities [7–10]. The degradation of HA_L to HA_S often leads to the generation of biologically active ECM fragment (HA_S, M.W. $\sim 1 \times 10^5$ to 1×10^4 Da) from the intact/large size ECM (HA_L, M.W. $\sim 7 \times 10^5$ to 1×10^6 Da), during periods of epidermal proliferation, differentiation and development, as well as following epidermal injury [7].

Age-related changes in the sizes of HA have also been reported [11]. Specifically, while HA_S appears to predominate in young (4-week-old) mouse skin, the amount of HA_L increases in older (52+ week-old) mouse skin [11]. Age-related declines in total HA production have also been documented in both rodent [11] and human aged skin [12]. In addition, alterations in HA metabolism are associated with reduced skin growth [13] and impaired wound healing [14], and/or delayed resolution of a variety of skin diseases [1–3]. All of these observations support the idea that both low levels of HA deposition and HA size modifications could underlie age-associated changes that occur during skin disease progression.

Interaction of HA with CD44 (a HA receptor) often induces unique downstream functions in many different cell types [15]. Our previous study showed that a CD44 deficiency is accompanied by a reduction in HA staining as well as marked alterations in keratinocyte proliferation, differentiation and altered barrier function in CD44 knock-out (k/o) mouse skin [16]. Downregulation of CD44 in cultured keratinocytes (using CD44siRNA) also significantly inhibits HA-mediated keratinocyte differentiation and lipid synthesis [16]. Together, these observations suggest the importance of both CD44 and HA for several key epidermal/keratinocyte functions. However, the mechanisms by which HA (HA_S vs. HA_L) and its receptor (CD44) contribute to the regulation of distinct keratinocyte functions (e.g., proliferation, differentiation, and permeability barrier recovery) in aged epidermis have not yet been determined.

A number of studies indicate that HA binding to CD44 promotes RhoGTPase signaling in a variety of cell types including keratinocytes [15,17–19]. RhoGTPases (e.g., RhoA and Rac1) belong to members of the Rho subclass of the Ras superfamily [20] that are known to cycle between an active GTP-bound state and an inactive GDP-bound state which transmit diverse signals from the cell surface to intracellular targets. They function as molecular switches that, in response to external stimuli, regulate key signaling pathways that in turn control a variety of downstream metabolic activities [21]. RhoA signaling is known to be important in keratinoctye functions [22–24]. Several enzymes have been identified as possible downstream targets for RhoA in cellular functions. One such enzyme is Rho-Kinase (ROK-also called Rho-binding kinase) which is a serine-threonine kinase that interacts with RhoA in a GTP-dependent manner [25–27]. RhoA-activated ROK participates in a number of HA/CD44-mediated cellular functions [28–31]. HA also promotes CD44 interaction with Rac1 signaling, leading to altered cytoskeleton-mediated cell functions [32,33]. In contrast to RhoA, different cellular targets have been identified as downstream effectors for Rac1 signaling. One such target is protein kinase N-γ (PKNγ) (also called PRK2) which belongs to a family of serine-threonine kinases known to interact with Rac1 in a GTP-dependent manner [34–36]. In keratinocytes, Rho-activated PKNγ has been found to be involved in Fyn/Src kinase-regulated cell–cell adhesion during Ca^{2+}-induced differentiation [37]. The results of our previous study indicated that HA promotes CD44-mediated Rac1-PKNγ kinase signaling and downstream effectors, including PLC-γ1-mediated Ca^{2+} mobilization and cortactin–actin interaction. This pathway, in turn, regulates keratinocyte cell–cell adhesion and differentiation [17]. Whether the binding of HA (HA_S vs. HA_L) to CD44 activates RhoA-ROK and/or Rac1-PKNγ signaling in keratinocytes is an additional focus of this investigation.

In this study we determined that changes in HA-size distribution are closely associated with marked alterations in epidermal proliferation and differentiation with deleterious consequences for the permeability barrier function in aged epidermis. A topical application regimen consisting of HA_S or HA_L or sequential HA ($HA_S \rightarrow HA_L$) treatment selectively restored keratinocyte activities (e.g., proliferation and/or differentiation), increased skin thickness and improved the permeability barrier function in aged skin. The fact that both HA_S and HA_L failed to correct the epidermal defects observed in aged CD44 knock-out (k/o) mice demonstrate the requirement of CD44 in HA (HA_S vs. HA_L)-mediated regulation of epidermal functions in aged mice. Since treatment of cultured human keratinocytes (CHK) or mouse skin with ROK (Y27632) or PKNγ (Ro31-8220) inhibitors effectively reduced the expected HA_S or HA_L-mediated changes in epidermal functions, both RhoA-ROK and Rac1-PKNγ play an important role in HA (HA_S vs. HA_L)-mediated regulation of keratinocyte activities and epidermal function. Taken together, this information could point to new HA signaling-based therapies for the treatment of age-related skin diseases.

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal rat anti-human CD44 antibody (Clone: 020; Isotype: IgG_{2b}; obtained from CMB-TECH, Inc., San Francisco, CA.) used in this study recognizes a common determinant of the CD44 class of glycoproteins. Polyclonal mouse anti-involucrin and polyclonal mouse anti-profilaggrin were purchased from Covance Inc. (Princeton, NJ) and Zymed Laboratories Inc (South San Francisco, CA), respectively. Mouse Anti-PCNA antibody and ABC peroxidase reagents were obtained from Caltag Labs (Burlingame, CA) and Vector Labs (Burlingame, CA), respectively. A panel of immune-reagents such as mouse anti-RhoA antibody, mouse anti-Rac1, rabbit anti-PKNγ, goat anti-ROK antibody and goat anti-actin antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For the preparation of polyclonal rabbit anti-HAS1 antibody, rabbit anti-HAS2 antibody, rabbit anti-HAS3 antibody, rabbit anti-Hyal-1, and rabbit anti-Hyal-2-specific synthetic peptides [~15–17 amino acids unique for the HAS1 or HAS2 or HAS3 or Hyal-1 or Hyal-2 sequences] were prepared by the Peptide Laboratories using an Advanced Chemtech automatic synthesizer (model ACT350). All polyclonal antibodies were prepared using conventional DEAE-cellulose chromatography and tested to be monospecific (by immunoblot assays).

2.2. HA preparations

Large HA (HA_L) (molecular mass ~700,000–1,000,000 Da) was prepared from Healon HA polymers (purchased from Pharmacia & Upjohn Company, Kalamazoo, MI) using gel filtration column chromatography-Sephacryl S1000 column. Small HA (HA_S) fragments (molecular mass ~27,000 Da) was obtained by digesting HMW-HA with bovine testicular hyaluronidase (PH20) according to the method described previously [9]. Briefly, intact healon HA polymers (500 mg) was dissolved in 50 ml of 0.1 M acetate buffer (pH 5.4) containing 0.15 M NaCl and digested with