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Human as the Ultimate Wound Healing Model: Strategies for Studies Investigating the Dermal Lipidome

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Abstract

Purpose of Review—Educate the reader of the multiple roles undertaken by the human epidermal lipidome and the experimental techniques of measuring them.

Recent Findings—Damage to skin elicits a wound healing process that is capped by the recreation of the lipid barrier. In addition to barrier function, lipids also undertake an active signaling role during wound healing. Achievement of these multiple functions necessitates a significant complexity and diversity in the lipidome resulting in a composition that is unique to the human skin. As such, any attempts to delineate the function of the lipidome during the wound healing process in humans need to be addressed via studies undertaken in humans.

Summary—The human cutaneous lipidome is unique and play a functionally significant role in maintaining barrier and regulating wound healing. Modern mass spectrometry and Raman spectroscopy based methods enable the investigation epidermal lipidome with respect to those functions.

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Conflict of Interest

Dayanjan S Wijesinghe, Urszula Osinska Warncke, and Robert F. Diegelmann declare that they have no conflicts of interest.

Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent

This article does not contain any studies with animal subjects performed by any of the authors. All human studies were carried out under the approval of the Institutional Review Board (IRB) of VCU-School of Medicine (IRB number 11087) and written informed consent was obtained from all participants.

Keywords

wound healing; bioactive lipids; analytical methods; sampling techniques; human

Introduction

Skin, the largest organ in the body, is protected by a continuous barrier of lipids that face the external environment. These lipids are a combination of those secreted by the sebaceous glands as well as those generated by the cells of the stratum corneum. The composition and distribution of lipids on the skin is unique to humans. Furthermore, skin lipids are often unique compared to lipids of internal tissues. In fact, the two key words used to characterize the human skin lipidome is “complexity and perversity”, where complexity is manifested by a large number of diverse lipid species and perversity is demonstrated by the uniqueness of skin lipids [1]. This is exampled by the fact that skin lipids contain significant amounts of both odd chain and branch chained lipids, a feature that is unique to the skin lipidome [1]. While there is significant spatial variability in the same person, the lipidome is also significantly altered during different stages of growth [2] and is also affected by environmental factors such as the different seasons, the skin microbiome and exposure to the elements [3–6]. Furthermore, significant variations exist between the skin lipidome of the different ethnic groups such as the Asian, African American and Caucasians [3]. This particular compositional spatial and temporal distribution of the lipidome found among humans are highly unique in the animal kingdom and cannot be replicated faithfully in any of the model organisms available for study. As such any studies that investigate the human dermal lipidome necessitates that those studies be undertaken using humans themselves as the model of choice. However, this requirement needs to be balanced with the potential for discomfort to subjects and minimizing invasive procedures wherever possible. In this regards, techniques such as micro sampling demonstrate significant advantages in the application towards studies investigating the human dermal lipidome. Furthermore, until recently, such detailed investigations of the human skin lipidome have not been feasible due to technological limitations. Technological advances in modern mass spectrometry based analytical methods has enabled micro sampling methods containing very small amounts of material to be used in the investigation of the variations in the human dermal lipidome during the wound healing process. Using these methods, as well as traditional techniques, a significant body of information has been derived with respect to the human skin lipidome.

Skin Surface Lipids

The primary sources of the human epidermal lipidome are the sebum and the cells of the stratum corneum. Triglycerides, free fatty acids, wax esters, squalene cholesterol esters and cholesterol constitute the primary human dermal lipids that are synthesized by the sebaceous glands [7]. The human sebum lipidome is especially unique in the fact some of the lipids such as squalene and wax esters are only found in the sebum secretions and nowhere else. Furthermore, the sebum derived skin lipidome is also unique in the presence of odd chain length and branched chain free fatty acids. An additional unique feature in the sebum derived human skin lipidome is the presence of Δ6 desaturase derived free fatty acids,

namely sapinic acid [16:1 Δ6] which constitute almost 25% of the total fatty acids and demonstrate a significant level of antimicrobial activity [8, 9]. Additional elongation and desaturation also gives rise to unique sebaleic acid [18:2 Δ5,8] which has recently been described as being important in neutrophil recruitment following transcellular conversion to 5-oxo-[6E,8Z]-octadecadienoic acid [10]. The primary functions of the human sebum lipidome include photoprotection, antimicrobial activity [e.g. sapinic acid], and delivery of fat soluble antioxidants to the skin surface as well as lipid specific pro and anti-inflammatory activity [7]. Alterations to the sebum lipidome has been implicated in multiple dermal human dermal health complications including acne, asteatosis, comedone, furuncles, comedones, carbuncles, sebaceous hyperplasia, seborrhea, seborrheic dermatitis and steatomas [7, 11]. As such detailed investigation into changes of the sebum lipidome associated with such disease states has the potential towards novel treatment options that specifically target the lipid balance of the sebum derived skin lipidome.

Stratum Corneum Lipids

The second and equally important source of the skin lipidome is derived from the stratum corneum [SC] and is primarily involved in the maintenance of epidermal permeability barrier and prevention of trans epidermal water loss [TEWL] [12–14]. These SC derived lipids are primarily composed of ceramides [50% by mass] with the remainder made up of free fatty acids and cholesterol [14–16]. These lipids fill in the gaps between the spaces of the keratinocytes in a “brick and mortar” type of structure where the dead and terminally differentiated keratinocytes acting as the bricks and the SC derived lipids act as the mortar [16]. As in the case of the sebum lipidome, human SC lipidome is unique in that a significant fraction of this lipidome is only found on the skin. In this regards, SC barrier lipidome composed of 4 different sphingoid bases N-acylated to 3 different fatty acyls creating a combination of 12 classes of ceramides [numbered CER1-12]. The sphingoid bases are primarily composed of 18 carbons and include sphingosine, sphingenine, phytosphingosine and 4-hydroxy-sphingosine (Table 1). The fatty acyls include non-hydroxy, omega-hydroxy and esterified-Omega hydroxyl fatty acids (Table 1). A majority of the sphingoid bases demonstrated antibacterial activity [8].

Signaling Lipids

In addition to the structural functions performed by the skin lipidome, a significant amount of signaling events are also mediated by the lipidome. We have demonstrated the importance of the sphingolipid ceramide-1-phosphate in the migration and proliferation of skin fibroblasts and demonstrated that this lipid species follow a temporal change during cutaneous wound healing in humans [17]. Additional studies by us and others have demonstrated the importance of eicosanoids in the mediating the signaling events during the wound healing process [17, 18]. Furthermore, we and others have demonstrated roles for sphingosine-1-phosphate in many of the aspects of wound healing biology [19]. Other lipids of relevance to the human dermal wound healing process include sphingophosphorylcholine [20], lysophosphatidic acid [21], protectins and resolvins [22].

Diseases Associated with Dysregulated Skin Lipid Metabolism

Dermatological research undertaken in the past few years have demonstrated a major role for the human dermal lipidome in the pathological conditions of the skin. In this regards, the role of ceramide metabolism with respect to atopic dermatitis is the most intensively investigated [15, 23–26]. For example, Yamato *et al* demonstrated that while the relative amounts of the major stratum corneum lipids remained unchanged, significant changes were observed within the ceramide class and the squalene, wax esters and triglycerides [24]. For example, the proportions of ceramides were demonstrated to be lower in the AD patients compared to their controls [24]. On the other hand, the sebaceous lipids were observed to be elevated in the AD patients compared to their controls [24]. The decrease in the ceramide content of the SC of the AD patients can be explained in part by the increased expression of sphingomyelin/glucosylceramide deacylase in the SC of AD patients [27]. This enzyme was demonstrated to hydrolyze sphingomyelin and glucosyl ceramides at the acyl site to liberate sphingophosphoroylcholine and glucosyl sphingosine which in turn lead to decreased production of SC ceramides [27]. In addition to the abnormalities in ceramides, significant changes were also observed in the cell membrane phospholipids in the epidermis of AD patients compared to their controls with a significant reduction observed in the phospholipid content of the epidermis of the AD patient [25]. These published studies demonstrate a close relationship between human skin lipid metabolism and AD. Psoriasis is another skin disorder that is due in part to lipid dysbiosis of the SC. In this regards, the generation of an abnormal skin ceramide composition leads to a disruption in the skin barrier function and elevated trans epithelial water loss [TEWL][12]. While the total ceramide content was demonstrated to remain unchanged, long chain ceramides containing ester linked fatty acids and those containing phytosphingoid backbones were demonstrated as being lower in psoriatic skin compared to normal skin [28]. These changes are attributed at least in part to decreased expression of the sphingolipid activation protein saposin [29, 30] which is a non-enzymatic component required for the hydrolysis of glucosyl ceramides. An inherited lipid related genetic disorder that leads to skin disease via dysregulated lipid metabolism is Gaucher disease. The disease is caused by a decrease in β-glucocerebrosidase and varies in clinical severity from asymptomatic to severe [31]. The decreased incidence of this enzyme manifests as an increase in the glucosyl ceramide and a decrease in the ceramide content [32] with a concomitant increase in the epidermal barrier function. Dry skin or Xerosis that often end up impairing barrier function is characterized by a deficiency primarily in the 6-hydroxy and 4-hydroxy backbone containing ceramides [33]. The fact that there is seasonal variation in skin ceramide content and the incidence in increases Xerosis in cooler seasons have been causally linked to each other [2]. Finally the most common of skin lipid mediated disorders would be acne. Alterations to the sebum lipidome has been heavily implicated in the outbreak of acne [11, 34–36]. Specifically, altered ratios between saturated and unsaturated fatty acids as well as altered amounts of specific fatty acids such as linoleic acid and the formation of squalene peroxides have all been linked to outbreaks of acne [37].

Pharmaceutical Modulation of the Skin Lipidome

Considering the primary roles played by the lipidome in the structure and function of the human skin, development of lipid formulations for both therapeutic and cosmetic purposes is

a highly active pharmaceutical industry [38, 39]. As such, several formulations include the use of ceramides or their precursors. Primary among those are formulations containing hydroxypalmitoyl sphingenine [Cetaphil, RestoraDerm Skin restoring Moisturizer], ceramide 1, ceramide 3 [Eucerin, CeraVe] and pseudo ceramides. Additionally, lipid blends such as epicerum [13] consisting of a blend of ceramides, cholesterols and free fatty acids are also used as cosmetic and barrier repair agents.

Sampling Methods for the Investigation of the Skin Lipidome

In order to explore different lipid components involved in human skin wound healing a variety of skin sampling techniques have been developed. The least invasive technique is called tape stripping [40]. One such process utilizes D-Squame® tape (CuDerm Corporation, Dallas, TX, USA) where the adhesive discs are applied to the skin using a set force and then ten or more successively samples are removed. The attached cells and lipid material on the disc are placed in an appropriate lipid extraction buffer followed by analysis of the lipids of interest [41]. Wound dressings are also a rich source of sampling material for research analyses of human wounds especially with respect to the signaling lipidome. A recent review by Widgerow *et al.*, describes the usefulness of collecting wound fluids under film dressings as well as analyzing components obtained from the discarded primary dressing [42]. This technique is especially useful for the study and progression of the healing of human chronic and burn wounds. The wound fluids and contents of the dressing can be extracted and the specific target analyte can be isolated and analyzed. Another useful technique is the suction blister model of wound healing to obtain interstitial fluid as well as the epithelial “roof” layer for analyses [18]. One useful instrument to create suction blisters on the inner forearm is the Negative Pressure Instrument [Electronic Diversities, Finksburg MD]. Alternatively a simple chamber attached to a standard vacuum pump can be used as well [350 mmHg for 1 to 2 hours]. The procedure is relatively painless and does not leave a scar. Creation of partial-thickness skin wounds using a variety of dermatomes has also been used to study human skin reepithelization [43]. The “donor site” type of wound can be photographed over time to quantify the process of reepithelization with or without the topical application of test materials. The wound surface can also be sampled using a sterile swab or Whatman filter paper and then analytes can be isolated and analyzed [44]. Punch biopsy is the classical procedure to obtain epidermal and dermal tissues for analyses. The recent review by Yang and Kampp provides a complete “how to” procedure to obtain skin biopsies for research and analyses [45]. The various layers in the full thickness specimen can be isolated and analyzed by histology as well as specific biochemical techniques to quantify proteins and bioactive lipids. In addition, the open wound created by the tissue punch can be covered with an occlusive dressing such as Opsite® (Smith and Nephew, Fort Worth, TX) and the wound fluid can be collected over time for analyses [46]. Furthermore, the process of wound contraction can be measured by using standardized photography and image analysis. One of the more recent and highly valuable techniques to study human wound healing has been the development of implantable and retrievable high-porosity Polytetrafluoroethylene tubes (012-01-2 PTFE; International Polymer Engineering, Tempe AZ) [47–51]. Typically the PTFE tubes can be implanted using an 18 g, 3.5 inch spinal needle in an anesthetized area in the inner aspect of the subject’s upper arm [52]. The

implants can be retrieved at sequential times such as 3, 5, 7 and 14 days after implantation and cut segments can be analyzed with respect to variations in the lipidome during the wound healing process (Figure 1). Using this technique, we have demonstrated that the sphingolipid ceramide-1-phosphate has a distinct variation during the human wound healing process and that it is likely acting as a master switch for the regulation eicosanoid signaling [17]. Additional and concurrent information can be gained by processing sections for histology, immuno-staining, matrix content, and with the use of an entropy-based automatic image analyzer system, specific cells and collagen deposition can also be quantified [53] as well as other signaling proteins that take part in the wound healing process [52].

Qualitative and Quantitative Analysis of the Human Skin Lipidome

Considering the fact that the lipidome is integrally involved in the function of the skin, its ability to heal and its various pathologies, the ability to quantitatively investigate its changes is highly describable. Furthermore, a majority of cosmetic products attempt to modulate the skin lipidome, and yet have ill-defined lipid compositions and is also worth investigating with respect to their claims in active lipid content [33, 39]. A significant body of information with respect to the quantitation of the skin lipidome has been obtained via analytical studies utilizing thin layer chromatography (TLC) [27, 28, 54, 54–59]. While TLC provides an affordable and low technology barrier method for analyzing the skin lipidome, it suffers from the inability to quantify individual lipid species. Furthermore the sensitivity of TLC towards determining the composition of the skin lipidome is also quite limited. Other analytical methods have been used over the years to obtain a more comprehensive understanding of the skin lipidome. These include p-nitrobenzoyl derivatization of skin ceramides followed by high-performance liquid chromatography with UV detection (HPLC-UV) [60] as well as gas chromatography coupled to mass spectrometry (GC-MS) [61, 61–63]. While better at quantitation of individual lipid species compared to TLC, these methods still suffer from limitations with respect to quantitatively capturing the full diversity of the skin lipidome. The most current technology for the analysis of the skin lipidome is atmospheric pressure ionization tandem mass spectrometry coupled to ultra-high performance liquid chromatography (UPLC API-MS/MS). Utilizing these methods, the human skin lipidome is currently being characterized extensively in many laboratories including ours [6, 17, 18, 56, 57, 64, 65, 65–70]. These studies have demonstrated unique changes in the skin lipidome during wound healing [17, 18, 67–70]. Application of such UPLC ESI-MS/MS methods have enabled our group to identify lipids that stimulate fibroblast growth in the presence of chronic wound fluid, a key requirement for cutaneous wound healing [19]. While, UPLC API-MS/MS methods are ideally suited for determination of the overall composition of the skin lipidome, these methods are not easily amenable for the investigation of the variations in the microscopic spatial distribution of the skin lipids. The most informative technique for determining the surface distribution of lipids of the skin surface is matrix associated laser desorption ionization mass spectrometry (MALDI-MS/MS) [71, 72]. While excellent in determining the spatial distribution of skin lipids, this method suffers from the primary drawback of being unable to distinguish between many of the isobaric lipid species and being limited in sensitivity for low abundant and low ionizing lipids. Furthermore, the method is only applicable for the investigation of excised samples.

Techniques that enable the investigation of the spatial distribution of the human skin lipidome *in vivo* has great value in point of care diagnostics. In this regards, Raman Spectroscopy based methods have demonstrated great promise and have been demonstrated to be applicable for in vivo investigation of the human skin lipidome [73–75]. In summary, considering the diversity and variability of the human skin lipidome, a single analytical technique is insufficient to obtain a comprehensive understanding. A combination of methods utilizing UPLC ESI-MS/MS, MALDI MS/MS and Raman spectroscopy is needed for the most comprehensive understanding of the variations in the human skin lipidome with respect to cutaneous wound healing and other lipid related skin pathologies.

Conclusions

The bioactive lipids in the skin provide a critical function in protecting the skin and come into play when the skin is damaged to facilitate the repair process. Because of the uniqueness and complexity of the human skin lipidome, it has not been possible to investigate it using animal and cell culture model systems. Now with technological advances employing micro sampling plus the development of advanced analytical instrumentation we can now extensively explore the skin lipidome in humans. These new avenues of research are enabling for a more in-depth understanding of the skin bioactive lipids and foster the possibilities for new translational research to help develop broader and multi modal therapeutic strategies to treat skin disorder and repair.

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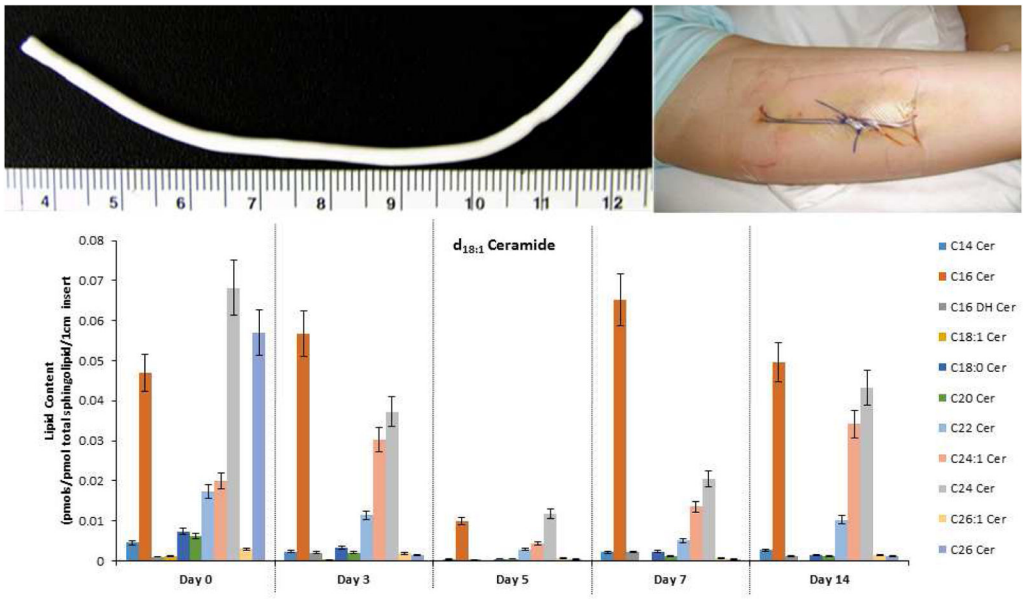
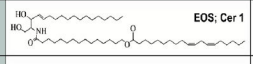
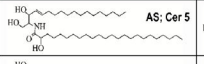
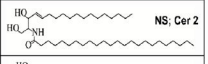
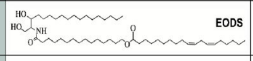
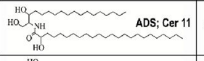
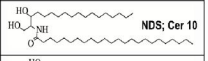
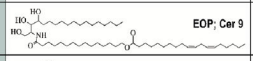
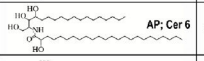
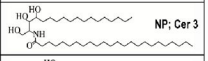
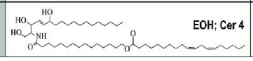
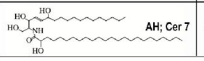
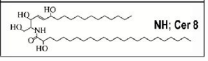


Figure 1. Alteration in the NS (Cer 2) ceramide profile during wound healing in humans

Lipids were extracted from a 1cm portion of PTFE implants inserted into the upper arm of healthy volunteers and removed on days 3, 5, 7 and 14. A 100 mm skin punch biopsy was used as the baseline (day 0). Lipids were extracted from those samples and subjected to targeted analysis via LC tandem mass spectrometry. The data shown is the average lipid content from 7 volunteers (n=7) normalized to total sphingolipids ± SD. The lipid content is depicted in pmol specific lipid/pmol total sphingolipids found in 1cm of PTFE insert.

Table 1

Human skin ceramide structural variants identified to date. The different structural isomers of ceramides are depicted together with their commonly used names demonstrating the diversity of the human skein ceramides.

	Esterified ω-hydroxy Fatty Acid **[EO]**	α-hydroxy Fatty Acid **[A]**	Non-hydroxy Fatty Acid **[N]**
Sphingosine **[S]**	 **EOS; Cer 1**	 **AS; Cer 5**	 **NS; Cer 2**
Dihydrosphingosine **[DS]**	 **EODS**	 **ADS; Cer 11**	 **NDS; Cer 10**
Phytosphingosine **[P]**	 **EOP; Cer 9**	 **AP; Cer 6**	 **NP; Cer 3**
6-hydroxy Sphingosine **[H]**	 **EOH; Cer 4**	 **AH; Cer 7**	 **NH; Cer 8**