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Original Article

Age related depth profiles of human Stratum Corneum barrier-related molecular parameters by confocal Raman microscopy *in vivo*

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ABSTRACT

In this study, stratum corneum (SC) depth profiles of hydrogen bound water molecule types, intercellular lipid (ICL) ordering, concentration of natural moisturizing factor (NMF) and keratin folding/unfolding properties are investigated *in vivo* for older (mean 50 years old) and younger (mean 29 years old) human skin using confocal Raman microscopy.

The results show that the SC of the older group is modestly thicker ($p < 0.1$), has more hydrogen bound water molecules at the depth 10–30% of the SC thickness ($p < 0.05$), has a higher ordered organization of ICL ($p < 0.1$) and higher concentration of NMF ($p < 0.05$) at the depth 20–40% of the SC thickness compared to the younger group. This study also reveals, that the hydrogen bonding state of water highly correlates with NMF and the lateral structure of ICL but not with keratin's folding/unfolding properties.

The presented results let suggest, that the decreased trans-epidermal water loss (TEWL) with increasing age cannot be sufficiently explained by only the increased SC thickness, but additionally by the increase of ICL ordering, higher NMF concentration and thus larger amount of hydrogen bound water molecules at the depth 20–40% of the SC thickness.

1. Introduction

Human skin undergoes chronic changes with increasing age and the aging process induces not only alterations on the appearances of skin, *e.g.* wrinkling and stiffness, but also on the inner status, *e.g.* biochemical and biophysical modifications of skin tissue. Most of the accumulated changes are the result of the influence of environmental oxidative factors, such as solar radiation and pollutants, which are known as potent enhancers of oxidative stress-related extrinsic aging (Kammeyer and Luiten, 2015). One of the common symptoms in skin is the decreased physiological function with increasing age, such as itching due to dryness, the susceptibility of skin damage and reduced recovery thereof (Boireau-Adamezyk et al., 2014a; Ghadially et al., 1995; Libertini, 2014). Hereby, one of the reasons for the aging process is the change of water, lipids and proteins in the stratum corneum (SC), the uppermost layer of the epidermis, which plays a critical role for maintaining the skin barrier function (Biniek et al., 2015; Blaak et al., 2011; Boireau-Adamezyk et al., 2014a, b; Egawa and Tagami, 2008; Ghadially et al., 1995; Lademann et al., 2012). Besides the SC thickness, the content and organization of intercellular lipids (ICL) (Boireau-Adamezyk et al., 2014a), and the water regulation in the SC has been considered as one of the crucial factors for maintaining the skin barrier function (Boireau-Adamezyk et al., 2014b; Egawa and Tagami, 2008; Imokawa et al., 1991; Lademann et al., 2012; Rawlings, 2014). Decreased percutaneous absorption has been shown in aged skin (Holmgaard et al., 2013; Roskos et al., 1989). This was suggested to be due to reduced amount of surface lipids, as the permeating substances were weakly lipid soluble, while permeation of substances with high lipid solubility was not decreased (Roskos et al., 1989). Decreased lipid content in aged skin, which is linked to the skin barrier function, has been reported by various groups (Boireau-Adamezyk et al., 2014a; Jensen et al., 2005; Rogers et al., 1996; Waller and Maibach, 2005). Saint Léger et al. (Saint Leger et al., 1988) reported decreased amount of sterol esters and triglycerides, while the amount of free fatty acids only changed slightly in aged skin. Similarly, Ghadially et al. (Ghadially et al., 1995) reported a decrease of the total lipid content, while ceramide, cholesterol and free fatty acids remained unchanged in aged skin. Biniek et al. (Biniek et al., 2015) suggested, that reduced amount

Abbreviations: SC, stratum corneum; CRM, confocal Raman microscopy; FFA, free fatty acid; TEWL, Transepidermal Waterloss; HWN, high wavenumber; ICL, intercellular lipid; AUC, area under the curve; DA, single donor–single acceptor; DDAA, double donor–double acceptor; DAA, single donor–double acceptor; NMF, natural moisturizing factor

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of lipid and lipid bilayers in aged skin increases the lateral packing order, which entails a decrease of water diffusion through the SC. The same group assumed that decreased NMF (natural moisturizing factor) expression in aged skin may reduce the hydration of the skin, leading to increase of the intermolecular forces between keratin fibers. This implies reduced mobility of the keratin and manifests as increased stiffness in aged skin. The skin barrier function has usually been determined by non-invasively measuring the Transepidermal Waterloss (TEWL) (Meguro et al., 2000; Wilhelm et al., 1991). Previous researches showed that TEWL is unchanged (Blaak et al., 2011; Firooz et al., 2012; Luebberding et al., 2013; Roskos and Guy, 1989; Sato et al., 2014) or decreased with increasing age (Berardesca and Maibach, 1990; Boireau-Adamezyk et al., 2014a; Cua et al., 1990; Ghadially et al., 1995; Kinn et al., 2015; Kobayashi and Tagami, 2004; Kottner et al., 2013; Tagami, 1972; Wilhelm et al., 1991), indicating that there is no significant degradations of skin barrier functions. However, a significantly slower relaxation of perturbed skin barrier function by acetone or tape stripping (Ghadially et al., 1995) and consequently slower relaxation of TEWL after occlusion of the skin has been reported in aged skin (Roskos and Guy, 1989). A possible explanation for the decrease of TEWL could be the increase of SC thickness of aged skin, which causes decreasing TEWL, despite of an age dependent degraded water regulation caused by disordering ICL (Boireau-Adamezyk et al., 2014a). An increase of SC thickness due to aging is however controversially discussed (Libertini, 2014; Waller and Maibach, 2005). In controlling water diffusion in the SC, there are two main factors. The first is the hygroscopic material, *e.g.* NMF, which consists of free amino acids, such as glycine, pyrrolidone-5-carboxylic acid, arginine, ornithine, citrulline, alanine, histidine, *etc.* and derivatives of amino acids, produced by the breakdown of filaggrin in keratinocyte cells and specific salts (O'Regan et al., 2010; Verdier-Sevrain and Bonte, 2007). The second is related to SC ICL, which form the main water transferring route (Kasting et al., 2003). The organizational orders of ICL lamellar structures play an important role in regulating the water evaporation through the skin (Elias and Menon, 1991; Imokawa et al., 1991; Imokawa et al., 1989; van Smeden et al., 2014; Williams, 1991). The ICL lamellar organization coincides highly with the regulation of water in the SC (Nakazawa et al., 2012). The hydrogen bound water molecule types in the SC, are highlighted in relation to pathological states of the SC and water controlling functions (Caussin et al., 2007; Egawa and Tagami, 2008; Meguro et al., 2000; Takenouchi et al., 1986; Vyumvuhore et al., 2015; Yadav et al., 2007).

Recently, confocal Raman microscopy has become an emerging tool in revealing the structure of ICL, proteins and water (Caspers et al., 2001; Gniadecka et al., 1998a; Gniadecka et al., 1998b; Lademann et al., 2009). In 1998, Gniadecka et al. (Gniadecka et al., 1998a; Gniadecka et al., 1998b) had attempted for the first time to differentiate the chronically aged and photo aged skin from intact skin by using Raman spectroscopy. The group revealed that the water of young and chronically aged SC were mostly in the bound forms and no significant changes between chronically aged and young skin were found, except a shift of the amide I band (1659 cm^{-1} for aged skin and 1663 cm^{-1} for young skin). However, the applied method was limited in revealing molecular structures of water molecules, because Raman signals greater than 3500 cm^{-1}, where O—H vibrations occurred, could not be acquired by the applied Raman instruments. Caspers et al. (Caspers et al., 2001) proposed a method to calculate the water concentration in the SC in the high wavenumber region (HWN, 2000–4000 cm^{-1}), by the ratio of the integrated area of the O—H vibration band (integrated from 3350 to 3550 cm^{-1}) and the keratin based Raman band (integrated from 2910 to 2965 cm^{-1}).

Differential scanning calorimetry was also used to measure the amount of bound water and revealed that the ICL organization is highly related to the bound water in the SC (Imokawa et al., 1991). Vyumvuhore et al. (Vyumvuhore et al., 2013a) showed that the different water types (primary bound water, partially bound water and unbound water) can be differentiated using Gaussian function based deconvolution of the O—H band (3100–3700 cm^{-1}). Boireau-Adamezyk et al. (Boireau-Adamezyk et al., 2014b), also categorized the water molecules into 3 types, by means of a perpendicular-drop-down cut-off integrating method of the O—H Raman band (3100–3600 cm^{-1}) and found minor differences in water types depth profiles in the SC of upper inner arms for two age groups. Recently our group developed a method to calculate the hydrogen bonding structures of water molecules in the SC by using a Gaussian deconvolution method, which is considered to be more accurate than the perpendicular-drop-down method. The new criteria to determine the hydrogen bonding states of water molecules by the ratio DA (single donor–single acceptor, weakly bound)/DDAA (double donor–double acceptor, strongly bound) water molecule types (Choe et al., 2014; Choe et al., 2016b) was adapted. Furthermore, the lipid's lateral packing orders could be more precisely calculated by taking account of the keratin influence on the lipid portion by analyzing the Raman spectra (Choe et al., 2016a, 2016c).

These stated new advances in *in vivo* analyzing SC's water and lipid organizations allow to study the differences of SC molecular structures between aged and younger skin more deeply. In the present study, the age-related changes of human SC are investigated by using confocal Raman microscopy *in vivo*. Especially the alterations of water, ICL and proteins are thoroughly investigated for older and younger skin *in vivo*, by focusing on the depth-dependent profiles of these substances in the SC.

2. Materials and method

2.1. Volunteers and sample preparation

11 healthy Caucasian volunteers (8 female, 3 male) aged from 23 to 62 years (average 37 years old) participated in this study. The volunteers were divided into two age groups: the "younger group" 23–34, mean 29 years old (3 male, 4 female) and the "older group" 45–62, mean 50 years old (4 female).

The volunteers were instructed not to utilize any skin care products on the forearms at least 72 h and not to bath or shower at least 4 h previous from the beginning of the experiments. After an acclimation time of 20 min, the skin area on the volar forearms was marked using a rubber barrier with the size of 2 × 2 cm^2. Then the intact skin was measured by confocal Raman microscopy (CRM) at more than 10 measuring points.

The volunteers had given their written informed consent. Approval for the measurements had been obtained from the Ethics Committee of the Charité – Universitätsmedizin Berlin and all the procedures complied with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2. Confocal Raman microscopy (CRM)

Confocal Raman microscopic measurements were performed using the skin composition analyzer dedicated for *in vivo/ex vivo* skin measurements (River Diagnostics, Model 3510, Rotterdam, The Netherlands). A 785 nm laser (20 mW on the skin surface) was used to analyze probes in the fingerprint (FP, 400–2000 cm^{-1}) and a 671 nm laser (17 mW on the skin surface) was used for analysis in the high wavenumber region (HWN, 2000–4000 cm^{-1}). Raman spectra were recorded from above the skin surface down to the depth of 40 μm with 2 μm increments. The exposure time for one measurement was 5 s in the FP and 1 s in the HWN region. The complete acquisition of a penetration profile for one measurement point lasted ≈3 min. The utilized doses of reference light (1.1 J/cm^2 for 785 nm and 0.2 J/cm^2 for 671 nm excitation) can be considered safe for human skin regarding local temperature increase (maximal 2 °C) (Akhalaya et al., 2014) and with regard to light- induced free radical generation (Darvin et al., 2007; Robert et al., 2015). The dose-dependent fluorescence photobleaching in the SC, without influence on Raman peak intensities