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Effects of sphingolipid extracts on the morphological structure and lipid profile in an in vitro model of canine skin

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ABSTRACT

Ceramides (CER) are essential sphingolipids of the stratum corneum (SC) that play an important role in maintaining cutaneous barrier function. Skin barrier defects occur in both human beings and dogs affected with atopic dermatitis, and have been associated with decreased CER concentrations and morphological alterations in the SC. The aim of the present study was to investigate the changes induced by three different sphingolipid extracts (SPE-1, SPE-2 and SPE-3) on the morphological structure and lipid composition of canine skin, using an in vitro model, whereby keratinocytes were seeded onto fibroblast-embedded collagen type I matrix at the air–liquid interface. Cell cultures were supplemented with SPE-1, SPE-2, SPE-3 or vehicle (control) for 14 days. The relative concentrations of lipids were determined by ultra-performance liquid chromatography coupled to mass spectrometry. The ultrastructural morphology of samples was examined by transmission electron microscopy. SPE-1 induced significant elevation in total CERs, CER[NS], CER[NDS], CER[NP], CER[AS], CER[AP], CER[EOS] and CER[EOP] subclasses, whereas SPE-2 induced a significant elevation in total CER, CER[AP] and CER[EOS] compared with control conditions. Ultrastructural analysis revealed an increase in lamellar-lipid structures in the SC of SPE-1-treated samples. The findings demonstrated that SPE-1 stimulates production of CERs, as shown by changes in lipid composition and ultrastructural morphology. Thus, SPE-1 contributes to the formation of a well-organised SC and represents a potential therapeutic target for improving skin barrier function in atopic dermatitis.

Introduction

The stratum corneum (SC) of the skin, the outermost layer of the epidermis, plays an important role in barrier function, by limiting the penetration of substances and pathogens, and restricting water movement into and out of the skin. The cornified cell envelope is located in the inner cytoplasmic membrane of corneocytes and is composed of a cross-linked protein layer, which provides structural and mechanical integrity to the cells (Nemes and Steinert, 1999; Candi et al., 2005) and confers a scaffold structure to the extracellular lamellar lipids (Bouwstra and Ponec, 2006). Assembly of the extracellular lamellar lipids is a crucial factor in maintaining permeability barrier function (Swartzendruber et al., 1987, 1989). Precursors of the extracellular lipid matrix, including phospholipids, glucosylceramides, sphingomyelin and cholesterol, are located in the lamellar granules of keratinocytes at the upper stratum spinosum and stratum granulosum, and originate from the Golgi apparatus (Downing et al., 1987; Candi et al., 2005; Feingold, 2007, 2011).

In human beings, the lipid matrix consists mainly of three lipid classes: ceramides (CER), fatty acids and cholesterol (Elias and Feingold, 1992). Ceramides, as the major constituent of extracellular lamellar lipids, play a key role in determining cutaneous barrier function and the water-holding capacity of the SC (Bouwstra and Ponec, 2006; Jungersted et al., 2008). Canine SC has a CER profile closely resembling that of human beings (Popa et al., 2010). These extremely complex skin CERs include numerous molecular subclasses consisting of a combination of sphingoid moieties, including sphingosine [S], dihydrosphingosine [DS], phytosphingosine [P] and 6-hydroxy-sphingosine [H], linked via an amide bond to a fatty acid moiety, which may be non-hydroxy [N], α-hydroxy [A], or ester-linked ω-hydroxy [EO]. At present, the following CER subclasses have

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been reported: CER[AH], CER[ADS], CER[AP], CER[AS], CER[EOH], CER[EOP], CER[EOS], CER[NH], CER[NDS], CER[NP] and CER[NS] (Masukawa et al., 2008; van Smeden et al., 2011; t'Kindt et al., 2012).

CER deficiency of the SC appears to be responsible, at least in part, for the cutaneous skin barrier defect and is recognised in both human and canine atopic dermatitis (AD) (Reiter et al., 2009; Shimada et al., 2009; Yoon et al., 2011; Nishifuji and Yoon, 2013). The dog has been shown to be a suitable model of human AD, because the development of clinical and immunological alterations in the disease resembles those in human beings (Santoro et al., 2013). Canine AD is defined as a genetically predisposed inflammatory and pruritic skin disease associated with immunoglobulin E antibodies against environmental and/or food allergens (Pucheu-Haston et al., 2015).

In canine AD, impaired cutaneous barrier function is associated with an increase in transepidermal water loss (TEWL) and, consequently, a reduction in skin hydration (Marsella and Samuelson, 2009; Hightower et al., 2010; Marsella et al., 2011). Increased TEWL has been associated with decreased CER levels and, in humans affected with AD, CER deficiency can be either a primary defect, the consequence of skin inflammation or both (Shimada et al., 2009; Santoro et al., 2015). Abnormalities of SC morphology have also been reported in skin from AD-affected dogs, as demonstrated by corneocyte disorganisation with wider intercellular spaces alongside an abnormal or incomplete lipid lamellae structure (Inman et al., 2001; Piekutowska et al., 2008; Marsella et al., 2010). There is increasing evidence that such defects might lead to excessive penetration by allergens and/or microorganisms, triggering an inflammatory process that can become chronic (Inman et al., 2001; Piekutowska et al., 2008; Ishida-Yamamoto et al., 2011). Recent studies have shown improvements in skin barrier function in dogs affected with canine AD following topical application and oral feed supplementation of preparations containing CERs, free fatty acids and cholesterol (Piekutowska et al., 2008; Popa et al., 2011, 2012; Jung et al., 2013).

In vitro models, known as skin equivalents (SEs), have been developed as an alternative to use of experimental animals. These bioengineered tissue substitutes consist of a dermal compartment, mainly consisting of collagen, which acts as a scaffold for primary cells of the epidermis (keratinocytes) and dermis (fibroblasts), thus replicating the cellular and structural properties of skin. SEs have been widely used in human skin biology research (Boyce and Williams, 1993; MacNeil, 2007), cutaneous irritation studies, toxicity testing (Welss et al., 2004; Park et al., 2010) and experimental modelling to test permeability and cutaneous absorption of different agents and formulations (Batheja et al., 2009; Tokudome et al., 2010). Although less well characterised, canine SE models have been developed (Barnhart et al., 2005; Magnol et al., 2005; Serra et al., 2007) and a canine SE model was validated for assessing the effects of topical oil formulations on skin morphology, functionality and changes in lipid composition (Cerrato et al., 2013). The aim of the present study was to investigate epidermal and dermal morphological structure and lipid profile composition in canine SEs exposed a number of lipid extracts rich in sphingolipids.

Materials and methods

Sphingolipid extracts

Three treatments were evaluated: sphingolipid extracts (SPE) 1–3 (Bioiberica SA). Each extract demonstrated a different lipid profile, but all were of animal origin. Compounds were emulsified in phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} to 0.1% (W/V) and serially diluted to the desired concentration.

Isolation and culture of canine skin cells

Dermal fibroblasts and keratinocytes were isolated from biopsies obtained from the abdominal skin of healthy dogs after routine surgeries unrelated to the present study. Biopsies were collected in the Veterinary Hospital of the Universitat Autònoma de Barcelona (UAB), after obtaining informed written consent from the owner. All experimental protocols were supervised and approved by the Animal Ethics Committee of the UAB.

Skin samples were cleaned, cut into small fragments and enzymatically dispersed as previously described (Llames et al., 2006; Cerrato et al., 2013). Skin fragments were incubated with 2 mg/mL collagenase type I solution (Sigma-Aldrich) in Dulbecco's Minimal Essential Medium (DMEM, Invitrogen) for 4–6 h at 37 °C until the dermis was totally digested. The cell-rich supernatant was centrifuged at 300 *g* for 5 min, washed with PBS and the resulting fibroblasts were incubated in a humidified atmosphere at 37 °C with 5% CO_2 in culture medium consisting of DMEM supplemented with 10% foetal bovine serum (FBS) and antibiotics (Invitrogen).

The remaining epidermal fragments were subsequently washed and digested with a solution of 0.05% trypsin and 0.02% ethylene diamine tetraacetic acid (EDTA; Invitrogen) for 30 min at 37 °C to obtain keratinocytes. These were filtered twice with cell strainers (100 μm and 40 μm pore size, respectively; BD Biosciences) and centrifuged at 300 *g* for 5 min to recover the cells. Keratinocytes were seeded onto collagen-coated flasks and cultured in a humidified atmosphere at 37 °C with 5% CO_2 in DMEM/F12 (3:1) (Invitrogen) supplemented with 10% FBS, 0.01 g/mL epidermal growth factor (Austral Biologicals), 5 g/mL insulin, 7 ng/mL cholera toxin, 0.4 g/mL hydrocortisone, 1.3 ng/mL triiodothyronine, 24 g/mL adenine (Sigma-Aldrich) and antibiotics. Culture medium was changed every 2–3 days and cells were used for the SE model between the second and fifth passages.

Skin equivalent model

To obtain a three-dimensional SE, a collagen gel biomatrix was developed in a transwell chamber (Corning) by adding 4×10^4/mL mature fibroblasts to a 1.5 mg/mL type I collagen solution (Sigma-Aldrich) (Cerrato et al., 2013). The collagen gel biomatrix was cultured for 5–7 days, when 5×10^5 keratinocytes were seeded onto it. After 24 h, SEs were lifted to the air–liquid interface and the medium was modified by including 1% FBS, 0.1% L-serine, free fatty acids (15 μM linoleic acid, 7 μM arachidonic acid and 25 μM palmitic acid), 50 μg/mL ascorbic acid, 1 μM DL-α-tocopherol-acetate and 2.4×10^{-5} M bovine serum albumin (Sigma-Aldrich). SEs were cultured for 48 h before replacing the medium with medium lacking FBS but with additional 30 μM linoleic acid.

On the third day after seeding the keratinocytes, SEs were cultured in medium supplemented with SPE-1, SPE-2, SPE-3 or vehicle only. The final concentration of compounds in the culture medium was 0.001% (W/V). Skin equivalents were cultured over a total period of 14 days and medium and treatments were changed three times per week.

Histological and ultrastructural studies

SEs were fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol and embedded in paraffin wax. Sections (4 μm) were stained using routine methods with haematoxylin and eosin (H&E) for histopathology. Ultrastructural studies were carried out by performing two different staining procedures. Firstly, samples were treated with paraformaldehyde–glutaraldehyde, then exposed to 1% (W/V) osmium tetroxide (TAAB) containing 0.8% (W/V) potassium hexacyanoferrate (III) (Sigma-Aldrich) for 2 h. Samples were dehydrated through a graded acetone series, embedded in Eponate 12 resin (Ted Pella) and polymerised for 48 h at 60 °C. Additionally, samples were treated with 2.5% glutaraldehyde, buffered in 0.1 M sodium cacodylate, then exposed to 0.2% ruthenium tetroxide in 0.1 M cacodylate (Sigma-Aldrich) for 1 h. After dehydration through a graded ethanol series up to 100% (W/V), samples were embedded in Spurr resin (Ted Pella) and polymerised for 48 h at 60 °C. Ultrathin sections (70 nm) from both staining procedures were cut with a diamond knife (45°; Diatome), mounted on copper grids (200 mesh) and contrasted with conventional uranyl acetate (30 min) and Reynold's lead citrate (5 min) solutions. Sections were visualised using a Jeol 1400 transmission electron microscope equipped with an Ultrascan ES1000 CCD camera (Gatan).

Lipid extraction and analysis

An ultra-performance liquid chromatography-mass/time-of-flight mass spectrometry (UPLC/TOF-MS) platform was used for profiling chloroform/methanol lipid extracts, providing coverage of glycerolipids, cholesteryl esters, sphingolipids and glycerophospholipids (Barr et al., 2012). Different treatments and SE samples were homogenised in the Precellys 24 homogeniser (Bertin Technologies) by mixing with chloroform/methanol (2:1, V/V) and sodium chloride (50 mM) (overall ratio 1:30:3, W/V/V), followed by protein precipitation. After brief vortexing, samples were incubated at –20 °C for 1 h. After centrifugation at 16,000 *g* for 15 min, the lower organic phase was collected and the solvent removed. The dried extracts were then resuspended in acetronitrile/isopropanol (1:1), centrifuged at 16,000 *g* for 5 min and supernatants were transferred to vials for UPLC-MS analysis on an Acquity-Xevo G2 Qtof mass spectrometry system (Waters Corporation). All UPLC analyses were performed in duplicate.