Cell surface glycans in the human stratum corneum: distribution and depth-related changes

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Abstract
During the formation of the stratum corneum (SC) barrier, the extracellular spaces of viable epidermis, rich in glycans, are filled with a highly organized lipid matrix and the plasma membranes of keratinocytes are replaced by cornified lipid envelopes. These structures comprise cross-linked proteins, including transmembrane glycoproteins and proteoglycans, covalently bound to a monolayer of cell surface ceramides. Little is known about the presence and distribution of glycans on the SC corneocytes despite their possible involvement in SC hydration, cohesion and desquamation. In this work, we visualized ultrastructurally and quantified the distribution of glycans on the surface of native and delipidated corneocytes. The cells were harvested at different depths of the SC, allowing us to define the relationship between the distribution of various glycans, proteoglycans and glycoproteins, and other changes occurring in SC. At the cell periphery, we found a correlation between the depth-related alterations of cornodesmosome glycoproteins and α-β-mannosyl and N-acetyl β-glucosamine-labelling patterns. Elimination of the terminal sugars, α-linked fucose and α-(2,3) linked sialic acid, was less abrupt, but also the initial extent of their peripheral distribution was overall lower than that of concanavalin A and wheat germ agglutinin lectin-detected glycans. Diffuse labelling of heparan sulphate glycosaminoglycans disappeared completely from the outermost corneocytes, whereas that of several simple carbohydrates could be detected at all SC levels. Our results suggest that specific glycan distribution may participate in the progressive changes of SC, as it evolves from the SC compactum to the SC disjunctum, towards desquamation.

KEYWORDS
corneocytes, desquamation, glycans, glycosaminoglycan, stratum corneum

1 | INTRODUCTION

The stratum corneum (SC) is the final product of epidermal differentiation. It is in constant renewal orchestrated by the cornification of living keratinocytes at the bottom and shedding of corneocytes at the skin surface. During cornification, the epidermis undergoes cellular and extracellular changes. At the cellular level, organelles such as nuclei and mitochondria are degraded, keratin filaments are compacted, and the phospholipid-rich plasma membranes are replaced by lipid envelopes, that is a monolayer of ceramides cross-linked to the cornified envelope (CE).¹,² CE is a 10- to 15-nm-thick, insoluble structure constituted by proteins deposited at the periphery of differentiated keratinocytes.
keratinocytes and recovered by a 5-nm-thick lipid envelope. Elements composing CE, including keratinocyte transmembrane proteins, are cross-linked via covalent bonds by transglutaminases 1, 3 and 5. SC may be considered as a depositary of the epidermis’ past, as it retains traces of the tissue activity preceding cornification. The extracellular spaces of viable epidermis, rich in glycans and notably in hyaluronic acid, are filled upon cornification with lipids organized in several molecular layers. This hydrophobic matrix highly contributes to the relative impermeability of the SC barrier.

Despite the rich literature concerning glycan expression on the surface of viable keratinocytes, the presence and distribution of glycans on the SC corneocytes are scarcely studied. Yet, sugar moieties may play an important functional role in the SC formation, cohesion and desquamation. On the other hand, deglycosylation of glucosyl ceramides is essential for the SC lipid maturation and permeability barrier formation.

Studies dealing with the identification of epidermal glycans used lectin-labelling technique and showed the presence of N-acetyl-D-glucosamine, sialic acid, α-D-mannosyl, α-D-glucosyl and O-galactosyl terminal sugars in the basal layer of the epidermis and N-acetylgalactosamine in the upper epidermal layers. Several epidermal proteoglycans bearing heparan sulphate glycosaminoglycans (GAGs), such as syndecans, epican, perlecan and glypicans, many of them showing differentiation-dependent expression, were detected in human epidermis. In their vast majority, these studies indicate a drastic reduction of these labellings within SC. However, such reduction was reported less pronounced in the case of in vitro-reconstructed epidermis. Moreover, detection of cell surface carbohydrates is largely altered in disorders of epidermal proliferation, characterized by incompletely differentiated SC.

Various hypotheses may explain these observations. (i) The absence of glycan moieties in the SC results from a considerable glucosidase activity at the bottom of the SC. For example, β-D-glucosidase that transforms glucosyl ceramides into ceramides and heparinase 1 that degrades heparan sulphates are both present at the interface between the living and cornified epidermal layers. (ii) Extracellular lipid matrix that covers corneocyte surface masks the glycan moieties and results in a false-negative outcome of the in situ detection. (iii) The occurrence of technical artifacts or bias cannot be excluded.

In the present study, we aimed at addressing this issue using an ex vivo approach, in which native and delipidated samples of normal human SC have been examined. The presence and distribution of various glycan moieties on corneocytes and changes in the glycan expression along the consecutive phases of SC maturation are herein described.

2 MATERIALS AND METHODS

2.1 Skin samples

Normal abdominal human skin from anonymous healthy female donors (aged between 20 and 50 years) was obtained during plastic surgery procedures according to the French regulations and Declaration of Helsinki act. A written consent was obtained from the patients.

2.2 Tape stripping

Strandard D-squame self-adhesive discs (CuDerm Corporation, Dallas, TX, USA) were applied onto the excised skin with a constant pressure for 10 seconds, then removed and stored in hermetic vials at −80°C. At least twenty consecutive tape stripings were performed on the same zone by the same operator to reduce variability. Tape stripings were also performed on delipidated skin. To this end, before each stripping step, a plastic ring was placed on the top of a sample and filled with a 1:1 mixture of chloroform/ether (v/v), left for 5 minutes before being removed, and the freshly delipidated surface was then tape-stripped.

2.3 Isolation of epidermal sheets and their delipidation

Superficial skin strips were obtained from the surgically excised tissue using a dermatome set at the depth of 0.8 mm. The epidermis was dissociated from dermis by placing the samples, dermal side down, into 2.5 mg/mL dispase in PBS (Gibco, Paisley, UK) for 4 hours at 37°C. Fragments of the isolated epidermis were divided into two lots. One was quickly washed in PBS and stored at −80°C. The other part was delipidated by immersion of the samples in a mixture of ether/chloroform (1:1) for 4 hours under constant stirring. Delipidated epidermis samples were also stored at −80°C. Isolated epidermal sheets were used for pre-embedding detection of glycans using histochemical methods.

2.4 Glycan- and protein-labelling agents

2.4.1 Dye

Ruthenium hexamine trichloride (RHT; Polysciences, Warrington, UK) is a chemical marker of GAG chains at cell surfaces.

2.4.2 Antibodies

Mouse monoclonal anti-CD44 HCAM (clone DF1485, diluted 1:10; Progen Biotechnik GmbH, Heidelberg, Germany); rabbit polyclonal anti-syndecan 1 (diluted 1:40; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); mouse monoclonal IgM from KM48 hybridoma cultures recognizing a highly glycosylated differentiation-dependent epidermal antigen (undiluted culture supernatant; Our Laboratory, Lyon, France); mouse monoclonal anti-corneodesmosin (diluted 1:100; Abnova, Jhongli City, Taiwan); mouse monoclonal anti-DSG1 p23 directed to the extracellular part of desmoglein 1 (undiluted; Amersham Biosciences, Little Chalfont, UK); mouse monoclonal anti-heparin/heparan sulphate (diluted 1:50; Bio-Rad, Kidlington, UK).
UK; mouse monoclonal anti-chondroitin sulphate (clone CS-56, diluted 1:50; Sigma-Aldrich, Saint Louis, MO, USA).

The primary antibodies were detected with goat anti-mouse or goat anti-rabbit immunogold reagents (5- or 10-nm colloidal gold conjugates diluted 1:10; BBI, Cardiff, UK).

Hyaluronic acid-binding protein (HABP) in its biotinylated form (diluted 1:40; Calbiochem, San Diego, CA, USA) was detected with the colloidal gold-conjugated goat antibody to biotin (10-nm granules, diluted 1: 10; BBI).

2.4.3 | Lectins

Colloidal gold-labelled lectins (10-nm granules) used in this study are as follows: Concanavalin A (Con A) that recognizes α-α-mannosyl and α-α-glucosyl monosaccharides, Maackia amurensis (MAA) that recognizes α-(2,3) linked sialic acid, peanut agglutinin (PNA) that recognizes N-acetylgalactosamine, Phytolacca americana (PWM) that recognizes N-acetylglucosamine, Sambucus nigra (SNA) that recognizes α-(2–6) linked sialic acid, Ulex europaeus agglutinin (UEA) that recognizes α-linked fucose and wheat germ agglutinin (WGA) that recognizes N-acetylgalactosamine and sialic acid. All lectins were purchased from EY laboratories (San Mateo, CA, USA) and used at 1:20 dilution.

2.5 | RHT staining for transmission electron microscopy

Small fragments of native and delipidated epidermis were fixed with 2.5% glutaraldehyde containing 0.7% RHT in sodium cacodylate buffer, then postfixed in 1% osmium tetroxide still in the presence of 0.7% RHT in the same buffer (Electron Microscopy Sciences, EMS, Hatfield, PA, USA). The samples were then dehydrated in graded ethanol and embedded in epoxy resins. For control purposes, delipidated epidermis samples were digested by the following glycosidases and glycosidase mixtures before being stained with RHT: (i) 10 μU of heparinase III in 100 mmol/L sodium acetate. 0.5 mmol/L calcium acetate buffer, pH 7.1, at 37°C for 24 hours; (ii) 500 mU of chondroitinase ABC in 50 mmol/L Tris-HCl buffer, pH 7.5, containing 50 mmol/L NaCl, 2 mmol/L CaCl₂, and 0.01% bovine serum albumin, at 37°C for 24 hours; (iii) 500 mU of hyaluronidase in 50 mmol/L sodium acetate buffer, pH 5, containing 75 mmol/L NaCl, at 37°C for 24 hours; (iv) consecutive digestions with hyaluronidase, as described, followed by a 24 hours digestion at 37°C with a mixture of 10 μU of heparinase III and 500 mU of chondroitinase ABC in 100 mmol/L sodium acetate, 0.5 mmol/L calcium acetate buffer, pH 7.1. All the enzymes and buffers were purchased from Sigma. The presence of the RHT staining was evaluated on ultrathin tissue sections routinely counterstained with uranyl acetate.

2.6 | Pre-embedding labelling with lectins and antibodies for transmission electron microscopy

Double-labellings with colloidal gold-labelled lectins (a direct labelling) and with antibodies followed by appropriate immunogold conjugates (indirect immunolabelling) were performed on native and delipidated D-squame strips. After labelling, the samples were fixed in 2% glutaraldehyde, washed, dehydrated in graded ethanol series and embedded in epoxy resins. Ultrathin cross-sections were counterstained with uranyl acetate before transmission electron microscopy (TEM) examination.

2.7 | D-squame labelling for scanning electron microscopy

The exposed surfaces of corneocytes collected on D-squame discs were labelled by a panel of antibodies and lectins. Labelling experiments were repeated on samples that were collected in eight adult female donors, aged 20–50 years. For indirect immunolabelling, the D-squame surface was saturated by a blocking solution (Aurion, Wageningen, the Netherlands) for 20 minutes, and then incubated for 1 hour at room temperature with a primary antibody. After a PBS wash, D-sqames were incubated with the secondary antibodies labelled with 10-nm colloidal gold particles for 45 minutes before being washed three times with PBS and, finally, with distilled water. For the direct labelling with lectins, D-sqames were incubated for 30 minutes with gold-labelled lectins than washed in PBS and distilled water. In all cases, the labelling signal was amplified using a silver enhancement kit (BBI), and then, samples were partially dehydrated in graded ethanol series (up to 95%), dried overnight at room temperature, and observed with a Quanta 250 FEI scanning electron microscopy (SEM) in low vacuum conditions (1 Torr) using secondary and backscattered electron detection modes.

To check specificity of the lectin labelling, three different strategies were applied: (i) deglycosylation of the corneocyte surfaces before labelling using PNGase F, O-Glycosidase, α-(3,6,8,9)-Neuraminidase, β-1→4-Galactosidase, β-N-Acetylgalactosaminidase (Sigma-Aldrich deglycosylation kit); (ii) saturation of the lectins with their respective saccharide inhibitors, before application onto D-squares; (iii) pre-incubation of corneocytes with unlabelled lectins before the application of gold-labelled probes. For indirect labellings, omission of the primary antibodies constituted the negative controls.

2.8 | Quantitative evaluation of the labelled cell surfaces

Measurements of the extent of cell margin labelling observed with SEM were carried out using ImageJ free online software on samples from the eight different donors with five markers at three SC depths (superficial, intermediate and deep SC). At least 10 cells per D-squame strip were evaluated. On each cell, the width of the marginal labelling zone was measured at four different points. The results were expressed as a mean width of the marginal zone ± standard deviation (SD) and compared using ANOVA one-way test.
3.1 Glycans at the corneocyte surface can be detected with RHT after SC delipidation

Ruthenium hexamine trichloride failed to stain corneocytes in native isolated epidermis (data not shown). Only after delipidation, a thin layer of RHT staining material appeared on the free surface of corneocytes (Fig. 1). The stain presented an approximately 10-nm-thick, deep-dark, uniform line superimposed over the lipid envelopes. The "cores" of corneodesmosomes exposed through mechanical disruption were also coloured (Fig. 1a). Ruthenium hexamine trichloride staining profile changed when the chemical was applied onto the samples predigested with enzymes targeting GAG. After digesting for 24 hours with heparinase III or chondroitinase ABC, that degrade heparan and chondroitin sulphates, a disruption of the cell coat stained with RHT was observed (Fig. 1b, c). No major change of the surface staining pattern was noticed after hyaluronidase digestion (not shown). Only after consecutive digestions with hyaluronidase followed by heparinase III and chondroitinase ABC, the RHT staining is no more present at the exposed surfaces (empty arrows). Bars = 100 nm.

3.2 Different glycan moieties are present on superficial corneocytes of delipidated SC in various distribution patterns

In the light of results obtained with RHT staining, the cytochemical studies of corneocytes were performed on delipidated tissues. Antibodies that recognize heparan sulphate and chondroitin sulphate GAG, as well as lectins recognizing specific sugar moieties, showed particular SEM labelling profiles on delipidated corneocytes from the SC disjunctum (Table S1). Each marker gave a reproducible labelling profile on various donors.

Antibodies to the protein cores of CD44, syndecan 1 and desmoglein 1 showed a very weak labelling dispersed at the corneocyte surface, with a slight tendency to cumulate at the cell margins. Labelling with the anti-heparan sulphate GAG antibody also followed this pattern, whereas antibody to chondroitin sulphate GAG and HABP failed to label superficial corneocyte surfaces (Fig. S1).

Antibodies to corneodesmosin and to the extracellular portion of desmoglein 1 showed a patchy labelling pattern concentrated at the corneocyte margins. The peripheral clusters visualized with the above-mentioned antibodies were mostly ovoid in shape with a longer diameter of 335±30 nm and a shorter one of 229±37 nm. Labelling of the cell margins obtained with Con A, MAA, PWM, UEA and WGA was highly reminiscent of that detected with antibodies to corneodesmosome proteins (Figs 2 and S1). However, Con A, PWM and WGA showed an additional diffuse labelling dispersed over the cell central plate of the cells (Fig. 2c). With PNA and SNA lectins, the labelling was absent or very weak and uniformly distributed over the corneocyte surface, respectively, with no particular labelling of the margins.

Transmission electron microscopy of vertical ultrathin sections of corneocytes double-labelled with lectins and corneodesmosin confirmed that the patchy labelling at the corneocyte extremities was mostly related to the presence of corneodesmosome structures.
whereas the central plate labelling was independent of any defined morphological features (Fig. 3a, b).

Enzymatic deglycosylation of the corneocyte surfaces with Sigma-Aldrich kit made impossible any subsequent lectin labelling, although it did not affect the detection of corneodesmosin. The use of lectin inhibitors considerably reduced the peripheral labelling intensity without completely blocking the central plate labelling (Fig. S2).

3.3 Changes in the labelling profiles of different markers at various SC depths

In situ delipidation, performed on excised skin samples, resulted in a diminished number of D-squame strips that we were able to obtain (10.3±2; mean±SD; n=8), when compared to the total number of strips necessary to completely eliminate the native SC (22±1.7). Instead, an increased number of corneocyte layers were harvested with each D-squame strip of the delipidated tissue.

Tape strips number two, n/2, and n, where n is the total number of strips collected from a given sample, were selected for labelling (Fig. S3). The most striking depth-related feature observed was the statistically significant decrease in the corneocyte margin labelling with lectins and antibodies, accompanying the displacement of the cells towards the SC surface (Fig. 4). In this respect, measurements of the peripheral labelling width showed that the dynamics of corneodesmosin, Con A and WGA elimination was similar, with decreasing linear trend curves’ slopes at 0.98, 1.11 and 1.03, respectively. Compared to that, corneocyte margins labelled with UEA and MAA lectins that recognize terminal sugars were thinner in the deepest SC but presented “slower” decreasing slopes at 0.47 and 0.52, respectively (Fig. 4). Heparan sulphate GAG labelling was weaker than that of Con A and WGA lectins on the margins of corneocytes from the deeper part of SC and tended to completely disappear at the surface of outermost cells (Fig. S3d–f). Labelling of the corneocyte central plates with antibodies to corneodesmosin and heparan sulphate GAG was weak and diffuse in deep SC, and absent in superficial SC. On the contrary, Con A, WGA, MAA and UEA showed diffuse central plate labelling of corneocytes throughout the whole SC depth. HABP and chondroitin sulphate antibody failed to label corneocytes, regardless of their position at different SC depths.

The dispersed distribution pattern of the proteoglycans’ protein cores was observed at all SC depths, although the labelling was more intense at the surface of the deepest corneocytes (data not shown).

4 DISCUSSION

Ruthenium hexamine trichloride staining procedure, which is a fairly specific technique to detect cell surface GAGs, allowed for corneocyte surface glycan’s visualization only after the elimination of intercellular lipids. Predigestion of SC with hyaluronidase alone...
did not modify the baseline RHT staining pattern, indicating that the dye targeted primarily cell surface GAGs other than HA. However, the successive actions of hyaluronidase followed by heparinase III and chondroitinase ABC allowed for the complete elimination of the cell surface carbohydrates stained with RHT. Our attempts to ultrastructurally localize HA using HABP failed to reveal this GAG either in the extracellular spaces of SC or at the surface of native and delipidated corneocytes (data not shown). HA is mostly processed by hyaluronidase 1 during the transition between SG and SC but is not completely degraded. In fact, Sakai et al. reported that the chain length of SC HA was 100 times lower then epidermal HA chains and that the binding activity of HABP to the shorter chains was dramatically reduced.

To precise the distribution of different glycans at the surface of corneocytes, we used the labelling technique on tape strips adapted for SEM. Cell surface delipidation before each consecutive tape stripping allowed us to label the exposed surfaces of corneocytes from the superficial, intermediate and deep portions of the horny layer. Immunogold labelling of heparan and chondroitin sulphate proteoglycans, such as CD44, syndecan 1 and desmosealin, showed the presence of these proteins all over the surface of corneocytes from the profound portion of SC. This may explain the observed continuous deposition of diffusible RHT dye over the cornified cell envelopes. The presence of proteoglycans in the lower SC may suggest their implication in the hydration of the SC compactum, with its obvious functional consequences. Another well-known capacity of GAGs is their interaction with other carbohydrates and proteins. Here, the potential partners within the extracellular spaces could be cell–cell junction glycoproteins and hydrophilic molecules involved in the SC processing. The degradation of corneodesmosomes is essentially under the control of two families of proteases, kallikreins and cathepsins, and their inhibitors. They are synthetized in the SC and are mostly concentrated in corneodesmosomes. Provided those glycan moieties could be remnants of glycoproteins that subsisted enzymatic degradation. Brysk et al. developed an in vitro assay in which delipidated but not native corneocytes were shown to aggregate. As this phenomenon was abolished by pretreatment of delipidated corneocytes with lectins, the authors concluded that cell surface sugars participate in the cell–cell adhesion process in the absence of the lipid spacer. These observations remain in agreement with our present results, as Con A, WGA and PWM lectins as well as proteoglycan and heparan sulphate GAG labelling covered the entire surface of delipidated corneocytes. In fact, an increased corneocyte cohesion can also be observed in situ, after a complete delipidation of the SC, when cornified lipid envelopes are shown to stick to one another.

Our results indicate that glycans do persist on the cell surfaces in the SC and are mostly concentrated in corneodesmosomes. Provided the importance of glycans for tissue hydration and their potential protective role against premature proteolysis, we suggest that these sugar moieties may participate in the maintenance of SC barrier function and in the regulation of SC desquamation.

**ACKNOWLEDGEMENTS**

Electron microscopy samples were observed at the Centre Technologique des Microstructures (CTµ), a Lyon Bio Image facility of the Lyon 1 University, France.
AUTHOR CONTRIBUTION

RA and AMM performed the research; FF and AP contributed to the design of the study along with MH, AMM and RA; RA, MH, AP and FF analysed the data; MH and RA wrote the manuscript. RA was a PhD fellow with l’Oréal (CIFRE fellowship).

CONFLICT OF INTERESTS

The authors have declared no conflicting interests.

ABBREVIATIONS

CE, cornified envelopes; GAG, glycosaminoglycan; HABP, hyaluronic acid-binding protein; HA, hyaluronic acid; RHT, ruthenium hexamine trichloride; SC, stratum corneum; SEM, scanning electron microscopy; SG, stratum granulosum; TEM, transmission electron microscopy.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1 Representative SEM images of superficial cornocytes labelled with various glycan, glycoprotein and proteoglycan markers

Figure S2 Examples of specificity controls of the SEM labelling profiles obtained with antibodies and lectins

Figure S3 Changes in the labelling profile of different markers according to the corneocyte location within the SC

Table S1 Labelling profiles of different lectins and antibodies at the surface of delipidated superficial human corneocytes