Lipid Rafts and Caveolae in the Terminal Differentiation of Epidermal Keratinocytes

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Abstract: Lipid rafts are cholesterol and sphingolipid-enriched plasma membrane domains, Caveolae represent a subclass of lipid rafts and the chief structural proteins are caveolins (caveolin-1, -2 and –3). Caveolae formation plays a major role in epidermal barrier permeability, regulating lamellar body secretion and terminal differentiation. Disruption of the epidermal barrier leads to protease-activated receptor-2 activation and an increased intracellular calcium resulting in lamellar body secretion. Caveolin-1 is transported *via* the lamellar bodies to the plasma membrane, inserted into lipid rafts and initiates caveolae formation. The insertion of caveolin-1 serves as a "brake" in lamellar body secretion and signals terminal differentiation in order to restore an efficient epidermal barrier.

Keywords: Caveolae, lipid rafts, lamellar body secretion, terminal differentiation.

INTRODUCTION

The epidermal barrier resides in the protective, semi-permeable stratum corneum (SC) that permits terrestrial life [1]. An intact SC is crucial to maintain a barrier that prevents the loss of fluids, electrolytes and other molecules from within the body and, at the same time, prevents penetration by microorganisms, toxic materials and UV radiation. SC permeability barrier function is provided by lipid bilayer lamellae surrounding apoptotic corneocytes, the so-called "bricks-and-mortar model" [2]. The intercellular spaces are filled with lipid lamellae (Fig. **1B**; "mortar") build from a mixture of ceramides, free sterols, and free fatty acids made by the secretion of lamellar bodies (LB) at the level of the stratum corneum/stratum granulosum (SC/SG) junction. LB originate from the tubulo-vesicular elements of the trans-Golgi network [3], where lipids and proteins are sorted for secretion [4].

THE DYNAMICS OF LAMELLAR BODIES SECRETION

LB secreted at the SC/SG junction, fuse with the apical plasma membrane (APM) of the outermost SG cell, creating thus a cholesterol/glycosphingolipid-enriched lipid raft-like domain. This secretion happens at low rates under normal conditions allowing a sufficient delivery of LB content, enough to maintain barrier function (Fig. **1**).

However, immediately following acute barrier abrogation, an orchestrated sequence of responses occurs rapidly to restore the barrier function to its basal level [5, 6]. Among these, the instant secretion (within 30 minutes) of the preformed LB from the outermost SG takes place. Application of either monensin or brefeldin A, known inhibitors of exocytosis and organellogenesis delay barrier recovery by affecting LB secretion and content respectively. While the signaling events that regulate LB formation/secretion are not yet fully understood, a decline in cation gradients across the epidermis (i.e. calcium and potassium) stimulates the initial secretion of LB that occurs in response to barrier disruption [7]. Nevertheless, the secreted lipids "dumped" from LB-fusion with the AMP at the SG/SC are processed into lipid bilayers by secretory phopholipase A2 (sPLA2), steroidsulfatase (SSase), acid sphingomyelinase (aSMase) and β-glucocerebrosidase (β-GlcCer'ase) in the SC. Surprisingly, we recently found that aSMase delivered to the SC interstices is expressed in the raft domain fraction of the epidermis (Fig. **2**).

THE LIPID RAFT HYPOTHESIS

Simons and van Meer back in 1988 described lipid rafts domains as dynamic, localized assemblies of cholesterol and sphingolipids within the plasma membrane [8]. Caveolae represent a subclass of those rafts and are enriched in caveolin proteins, a family of three (cav-1 to -3) small molecular weight (18-24 kDa) proteins, that cycle between the trans-Golgi network and the plasma membrane [9] (i.e. the natural flow of LB). Caveolin proteins form homo- and hetero-oligomers, which directly bind to cholesterol, required for the insertion of caveolae into membranes [10]. Cav-1 possesses a 'scaffolding domain' that interacts with signal transduction molecules [11]. Not only is this domain required to form multivalent homo-oligomers with other cav proteins, but it also mediates the interaction of cav-1 with non-cav proteins, such as the G-subunits, Ha-Ras, Src family kinases and eNOS [12-15]. Consequently, cav-1 acts as molecular 'Velcro' where signal transduction complexes are bound in the inactivated state [16]. Among the 3 cav proteins, cav-1 has been reported to be essential for caveolae formation. However, neither caveolin (1-3) knockout (-/-) mice show major abnormalities in their phenotype [17]. We

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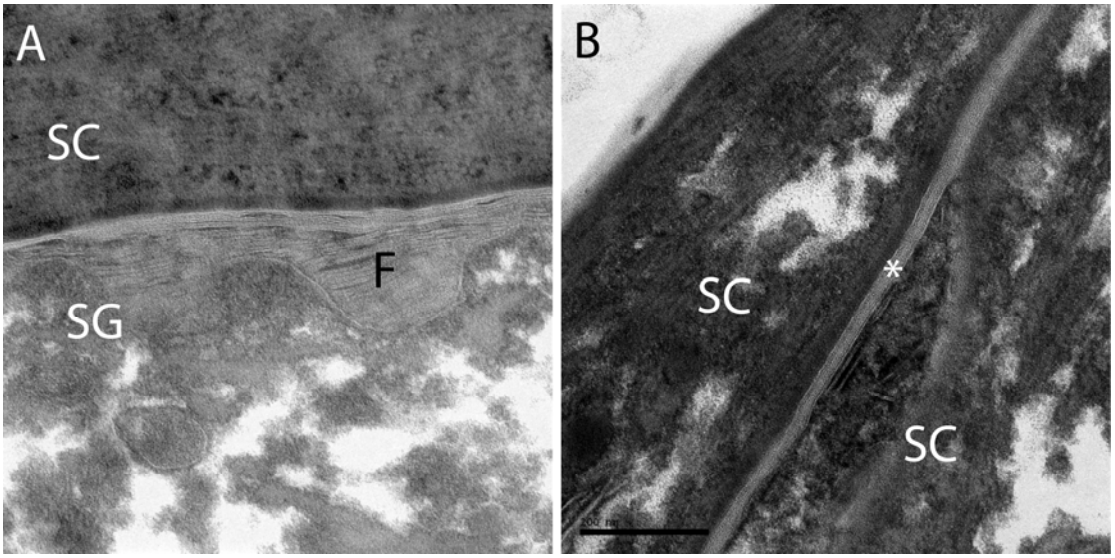

Fig. (1). Structure of the SC and the SC/SG interface. (A) fusion (F) of a lamellar body with the apical plasma membrane of stratum granulosum cell (SG). (**B**) lipid lamellae organized in bilayers between 2 stratum corneum (SC) cells. Magnification bar: 200nm.

addressed the importance of cav-1 *vs* cav-2 and -3 in cav-1-/- mice and we investigated whether the other cav proteins compensate for cav-1 loss. We performed western immunoblotting for both caveolin (2 and 3) proteins on protein extracts (lipid rafts -LR- and cytoplasmic -C- fractions) from both cav-1 -/- and +/+ littermates prior and following permeability barrier abrogation. The later was performed by cellophane tape stripping of the SC as described elsewhere [18]. Cav-2 proteins were undetectable by western immunoblotting of the cytoplasmic fraction (Fig. **2B**) consistent with the accessory function of cav-2 as a binding partner of cav-1 [19, 20]. In addition, it has been suggested that phospho-cav-2 is degraded in the absence of cav-1 suggesting that cav-1 -/- mice constitute a virtual knockdown of cav-2 [21]. Yet, western immunoblotting (cytoplasmic fraction) shows an increased expression of cav-3 (i.e. specifically after barrier abrogation) in cav-1 mice, which may in part compensate for the loss of cav-1 (Fig. **3B**).

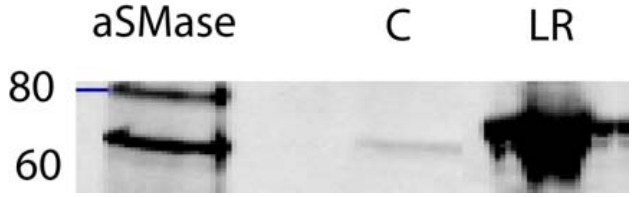

Fig. (2). Acid sphingomyelinase (aSMase) is expressed in the lipid raft fraction of mouse epidermis. Subcellular fractions from C57BL/6 mice epidermis were isolated using Ready Prep Protein Extraction Kits, according to the manufacturers' protocol. Western immunoblotting was performed for aSMase on cytoplasmic (C) and lipid raft (LR) fraction.

THE CAVEOLA BRAKE HYPOTHESIS I: CAVEOLA FORMATION REGULATES LAMELLAR BODIES SECRETION

Many functions have been attributed to rafts, from cholesterol transport [22], endocytosis [23] and signal transduction [24], but the role of caveolae, specialized LR in LB formation and secretion is unclear. It is generally recognized that influx of Ca^{2+} and Cl^- ions into epidermal keratinocytes through ionotropic receptors plays a crucial role in cutaneous barrier homeostasis [25]. LB secretion is regulated by the extracellular Ca^{2+} content of the upper epidermis, which is altered following permeability barrier disruption [7]. Hence, there has been an interest in the role of caveolae in regulating intracellular Ca^{2+} concentration [26]. As cav-1 may regulate both Ca^{2+} entry into cells and Ca^{2+}dependent signal transduction, caveolae may contribute to the regulation of LB secretion at the SG layer. Cav-1 colocalizes to LB on immunoelectron microscopy. Therefore, caveolae may play a role in LB assembly, trafficking, and/or function [27]. But the mechanism relating LR/caveola formation and how these structures modulate LB trafficking is still unknown. Exocytosis of LB is the process whereby cytosolic LB vesicles fuse with the plasma membrane (Fig. **1**), incorporating vesicle proteins and lipids into the APM and releasing their content at the SC/SG interface. We recently assessed the morphological changes in APM dynamics by quantitative electron microscopy of hairless mouse epidermis both prior and following acute barrier abrogation. Application methyl-β-cyclodextrin (MβCD) that disrupt nascent LR, as they are formed in parallel with LB secretion, delays barrier recovery and, significantly reduces the number of APM invaginations created by LB fusion. In contrast, accelerated rates of barrier recovery were observed in cav-1 -/- mice, which correlated with both increased LB secretion content and the formation of giant areas of secretion in cav-1 -/- *vs* +/+ mice. We concluded that the dynamics of LB secretion and fusion with the APM are largely dependent upon permeability barrier status, and can be modulated either by disrupting lipid rafts or deleting cav-1 [28]. Even if absence of a cav-1 accelerates barrier recovery, it also favors the development of both epidermal hyperplasia [29] and non-melanoma skin cancer [29] and is also known to negatively regulate epidermal proliferation [16, 29]. We found that lipid raft formation was increased in psoriasis and others reported a downregulation of cav-1 expression in the same disease [30]. An essential

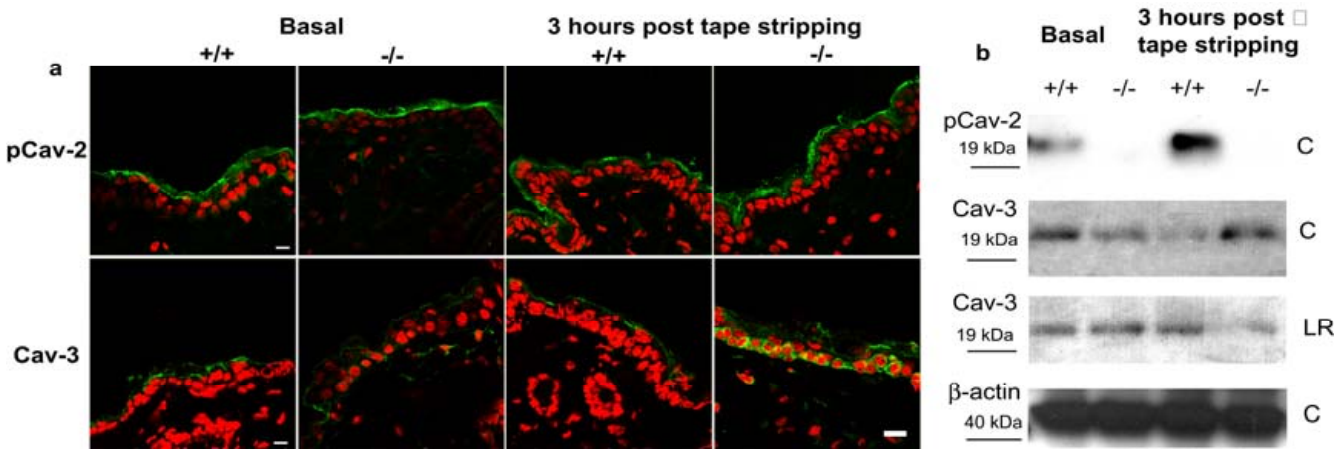

Fig. (3). The supremacy of caveolin-1: caveolin-3 but not caveolin-2 partially compensates for the loss of caveolin-1 in knockout animals. Immunohistochemical analysis of cav-2 and cav-3 was performed in cav-1 knockout (ko; -/-) animals and wild type (wt; +/+) littermates both prior and 3 hours following barrier abrogation. Only cav-3 was upregulated after barrier disruption in ko animals (**a**). This increase was confirmed by western immunoblotting and found to localize to the cytoplasm (**c**) in comparison to the LR domains (**b**). Contrariwise, cav-2 increases 3 hours post-barrier insult in wt animals but was undetectable by western immunoblotting in cav-1 ko mice. Sample loading control using beta-actin antibody was only performed for the cytoplasmic fractions.

issue that remains unresolved is how exocytic proteins and protein complexes are spatially regulated and whether these mechanisms account for LB secretion in the skin. Soluble N-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptors (SNAREs) are probable mediators of membrane fusion. Recent studies suggest that LR with their SNAREs constitutive proteins play an essential role in the regulated exocytosis pathways [32]. The association of SNAREs with LR in keratinocytes may act to concentrate these proteins at defined sites of the PM and drives LB secretion. Ichthyosis associated with arthrogryposis, renal tubular dysfunction and cholestasis (ARC) syndrome is due to mutation in VPS33B gene involving SNARE proteins family, results in an ichthyosiform phenotype [31]. In parallel, a necessary negative feedback ("brake") to secretion is necessary to transform the secretory granulocyte into an anucleated corneocyte. This “brake” is provided by caveola formation within LR.

We therefore hypothesized that cav-1 may play a role in the terminal differentiation process of epidermal keratinocytes, as cav-1 acts as molecular "Velcro" to nucleate the formation of signal transduction complexes, holding these molecules in the off state [16]. Cav-1 actually travels from the cytosol, carried by LB, and is incorporated into the APM by the fusion of the LB during the secretion process. This traffic creates the raft/caveolae domains within the APM of the outermost SG cell inducing thus the apoptotic transformation of the later into a corneocyte, in other word the terminal differentiation of a keratinocyte.

THE CAVEOLA BRAKE HYPOTHESIS II: CAVEOLA FORMATION REGULATES TERMINAL DIFFERENTIATION

At the SG/SC the outermost granular cells undergoes a programmed cell death, a crucial pace resulting in the production of corneocytes and the integration of the cornified envelope (CE) with the extracellular lipid matrix. Following the total secretion of the LB, the nuclei of the outermost SG cell is defragmented and transglutaminase starts cross-linking the cytosolic proteins to the plasma membrane to from the CE. Apoptosis is thus intrinsic and programmed in the epidermis and keratinocytes have all of the needed elements to undergo or prevent the apoptotic process [32]. The events that orchestrate SG keratinocytes transformation into SC cells has not been totally elucidated but recent evidence clearly suggests that apoptotic terminal differentiation is different from classical apoptosis [33]. We recently found that tape stripping of the SC alone induces a wave of cornification at the SG/SC interface that could be delayed by inhibiting several pathways, namely: SC serine protease (SP), protease-activated protease (PAR)-2, caspase-14 and caveolin-1 [34]. Others have found that DNase1-like 2 (DNase1L2) is preferentially expressed in the suprabasal levels of the epidermis and gene expression knockdown by siRNA inhibited terminal differentiation in human skin equivalents [35].

The SP/PAR-2 pathway suggests that the SC signals its own renewal by regulating SP activity. Thus, SP from the SC activate PAR-2, which may signal terminal differentiation together with LB secretion arrest [18]. PAR-2 ko demonstrate higher levels of proliferation (PCNA assay), suggesting enhanced epidermal proliferation. In addition, recent data from our group [18] and others [36] suggest that PAR-2 is localized to the LR membrane domains and thus could be implicated in the spatio-temporal distribution of raft domains by organizing the cytoskeletal proteins of keratinocytes. Using the cholera toxin assay to assess LR, PAR-2 -/- mice demonstrate abnormally increased LR formation both under basal and barrier abrogation conditions. It is well known that PAR-2 activation increases intracellular Ca^{2+}, which may in turn produce LB secretion/dumping, the formation of caveolae domains by LB trafficking and the signaling arrest that permits the transformation of the outermost SG cell into the apoptotic corneocyte (Fig. **4**).

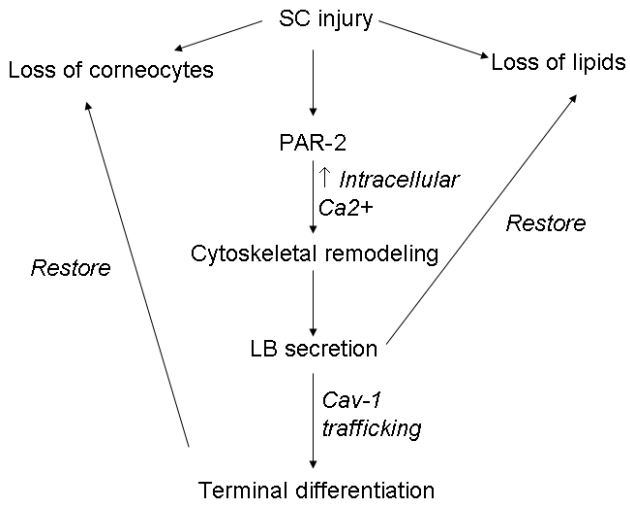

Fig. (4). The caveola brake hypothesis.

modulates the cytoskeleton. Consequently agonist peptide application upregulates tight junctions formation in cav-1 +/+ cells while cav-1 -/- cells remain completely detached (Fig. **5**). From these preliminary data, one could assume that LR/caveolae should play also a role in the formation of tight or even adherens junctions within keratinocytes. However more studies should be performed to more delicately address this issue.

CONCLUSION

Many functions have been attributed to lipid rafts/caveolae [22-24], and recently a role of caveolin-1 in lamellar body assembly, trafficking and function was suggested [27]. This could indicate a role of cavolin-1 and caveolae formation in epidermal barrier permeability homeostasis. Studies in methyl-β-cyclodextrin-treated mice show that disruption of lipid rafts leads to alterations in plasma membrane dynamics necessary for adequate lamellar body secretion at the SG/SC interface. In addition, studies in caveolin-1 knockout mice and monensin-treated mice

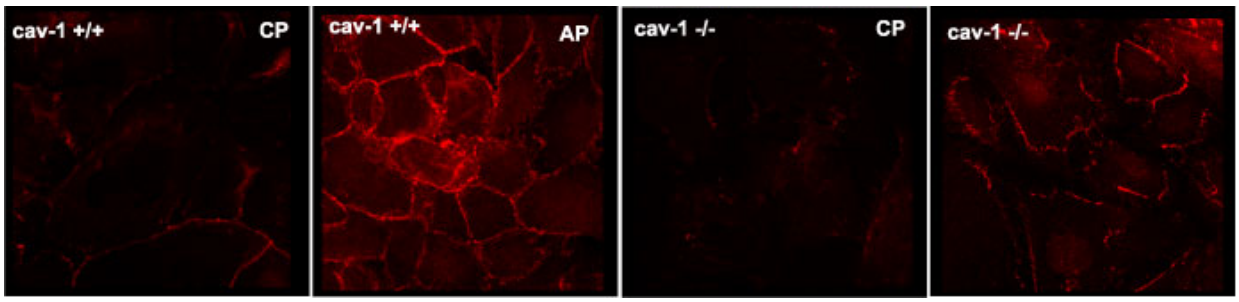

Fig. (5). Caveolin-1 regulates PAR-2-dependent cellular adhesion. Compared to control peptide (CP) treatment, PAR-2 agonist peptide (AP) increases tight junction formation in cav-1 +/+ assessed by immunostaining for zonula occludin 1 (ZO-1). contrariwise, cav-1 -/- cells are unable to establish cell to cell membrane contact.

CAV-1 AND JUNCTION FORMATION IN EPIDERMAL KERATINOCYTES: FUTURE TERRAIN OF INVESTIGATION

Increasing evidence suggests that lipid raft and caveolae play an important role in the regulation of membrane–cytoskeleton interactions [37]. Several cytoskeleton modulators such as, PAR-2 (see above), phosphatidylinositol 4,5-bisphosphate or Rho GTPases, and cytoskeletal proteins such as actin and vimentin are associated with rafts [38]. In epithelial cells, the structural protein of tight junctions occludin, and the peripheral membrane protein zonula occludens (ZO)-1 that links tight junctions to the actin cytoskeleton, has been found to localize into raft domains, and part of the occludin pool co-immunoprecipitates with caveolin-1 [39]. In addition, caveolin-1 may constitute an early and critical modulator that controls signaling pathways leading to the disruption of tight junction proteins [40].

We investigated whether Caveolin-1 is implicated in tight junction formation by addressing, among others, the expression of zonula occludens-1 (here shown) in both cav-1 -/- and +/+ cells. To modulate intracellular calcium we applied PAR-2 agonist peptide (SLIGRL) *vs* scrambled control peptide (IGLRLS) on both confluent cav-1+/+ and -/- cells during 30 minutes. In addition to the increase in intrecellular Ca^{2+}, PAR-2 activation by SLIGRL agonist also demonstrate the importance of caveolin-1insertion into lipid rafts and caveolae formation in barrier restoration. Next to an important role of caveolae in lamellar body secretion and terminal differentiation, preliminary data also suggest a role in establishing cell-cell contacts and adherens junction formation.

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