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Research Article

HaCaT keratinocytes exhibit a cholesterol and plasma membrane viscosity gradient during directed migration

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ABSTRACT

Keratinocyte migration plays an important role in cutaneous wound healing by supporting the process of reepithelialisation. During directional migration cells develop a polarised shape with an asymmetric distribution of a variety of signalling molecules in their plasma membrane. Here, we investigated front-to-back differences of the physical properties of the plasma membrane of migrating keratinocyte-like HaCaT cells. Using FRAP and fluorescence lifetime analysis, both under TIR illumination, we demonstrate a reduced viscosity of the plasma membrane in the lamellipodia of migrating HaCaT cells compared with the cell rears. This asymmetry is most likely caused by a reduced cholesterol content of the lamellipodia as demonstrated by filipin staining. siRNA-mediated silencing of the cholesterol transporter ABCA1, which is known to redistribute cholesterol from rafts to non-raft regions, as well as pharmacological inhibition of this transporter with glibenclamide, strongly diminished the viscosity gradient of the plasma membrane. In addition, HaCaT cell migration was inhibited by glibenclamide treatment. These data suggest a preferential role of non-raft cholesterol in the establishment of the asymmetric plasma membrane viscosity.

Introduction

Cell migration contributes to a multitude of physiological as well as pathophysiological processes, including development, wound healing, inflammation, and tumour dissemination. To migrate directionally, cells develop a polarised shape accompanied by cytoskeletal reorientation and a polarised redistribution of signalling proteins and lipids in the plasma membrane. The most prominent example in lipid polarisation is the lateral asymmetric distribution of PIP_3, which is generated by the interplay between locally activated PI3K and PTEN or SHIP-1 in *Dictyostelium* or neutrophils, respectively [1]. The role of other lipid components or physical membrane properties on cell migration is less well understood. Furthermore, there are very few investigations analysing the overall physical membrane properties of migrating cells which are highly influenced by the lipid composition. Ghosh et al. examined the viscosity of the plasma membrane of migrating endothelial cells [2], eliciting a slightly enhanced migration by stiffening the

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Abbreviations: ABC, ATP binding cassette; CLSM, confocal laser scanning microscopy; DiI, 1,1′-di-octa-decyl-3,3,3′,3′-tetra-methyl-indo-carbocya-nine perchlorate; FAST DiO, 3,3′-dilinoleyloxacarbocyanine perchlorate; FLIM, fluorescence lifetime imaging microscopy; FRAP, fluorescence redistribution after photobleaching; IRF, instrument response function; NBD-PC, 1-palmitoyl-2-(6-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl)-*sn*-glycero-3-phosphocholine; TIRF, total internal reflection fluorescence.

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membrane with α-tocopherol and an inhibited migration by reducing the microviscosity upon lyso-lipid incorporation. In a follow up publication, this group showed that these cells reveal a front-to-back gradient of plasma membrane microviscosity with an enhanced viscosity at the front of the migrating endothelial cells using FRAP and fluorescence polarisation experiments [3].

Cholesterol strongly influences the physical membrane properties [4]. The cholesterol content of the plasma membrane of a cell is regulated by the cellular cholesterol biosynthesis as well as its uptake from and release to serum lipoproteins. Release of cholesterol to serum lipoproteins is facilitated by the plasma membrane cholesterol transporters ABCA1 and ABCG1, which presumably mediate a transversal, ATP-dependent transport of cholesterol from the inner to the outer membrane leaflet [5]. Expression of functional ABCA1 was shown to alter the fluidity of plasma membranes [6]. Furthermore, studies using cholesterol-enriched macrophages, which play a role in atherosclerosis, show not only that functional ABCA1 inhibits migration of these macrophages due to an enhanced fraction of cholesterol in their plasma membrane [7], but also that deletion of ABCA1 and ABCG1 leads to an inhibited macrophage mobility [5].

Cutaneous wound healing depends on the recruitment of fibroblasts, endothelial cells, and keratinocytes [8], the migration of the latter determines the process of reepithelialisation [9]. Keratinocytes typically migrate in cell assemblies, and cells at the edge form pronounced lamellipodia. Among other factors, keratinocyte migration is stimulated by activation of the EGF receptor [10]. Here, using FRAP and fluorescence lifetime measurements under total internal reflection (TIR) illumination, we show that migrating keratinocyte-like HaCaT cells exhibit a polarised cholesterol distribution when stimulated with EGF, leading to a reduced microviscosity of their lamellipodia compared to the cell rears. Pharmacological inhibition of the cholesterol transporter ABCA1, which is expressed in HaCaT cells [11], diminished cell migration tested by a scratch assay and reduced the microviscosity gradient in polarised HaCaT cells. In addition, after silencing ABCA1 with siRNA, no plasma membrane microviscosity gradient was observable in polarised HaCaT keratinocytes.

Materials and methods

Cell culture

The spontaneously immortalised, untransformed human keratinocyte cell line HaCaT was kindly provided by Dr. U. Anderegg (Department of Dermatology, University of Leipzig, Germany). The cells were maintained in Dulbecco's modified Eagles medium with 1 g/l glucose, supplemented with 10% (v/v) foetal calf serum (FCS), 1% glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all PAA Laboratories, Pasching, Austria) and cultured at 37 °C in an atmosphere of 5% CO_2. For passaging, cells were washed, incubated for 10 min with PBS supplemented with 0.8 mM EDTA at 37 °C and trypsinised. For measurements, cells were seeded onto glass coverslips in 35-mm cell culture dishes.

Transfection of HaCaT cells

HaCaT cells were transiently transfected with Lipofectamine 2000 (Invitrogen). For cotransfection, siRNA and plasmid DNA transfection solutions were prepared separately in OptiMEM (Invitrogen) with 500 ng DNA and 25 pmol siRNA per 1.5 µl Lipofectamine 2000. The final concentration of siRNA against ABCA1 or ABCG1 (Supplementary Table 2, stealth siRNA, containing 3 different sequences at equal portions, Invitrogen) was 25 nM siRNA and 500 ng/ml plasmid DNA. After transfection of HaCaT cells, we used media devoid of antibiotics to enhance cell viability; however we observed no change in transfection efficiency in the presence or absence of FCS in culture medium.

Fluorescence redistribution after photobleaching measurements under TIR illumination (TIR/FRAP)

The fluorescence redistribution after photobleaching (FRAP) experiments were done 1 day after seeding, when no additional activation of the cells was performed. To investigate the effect of defined concentrations of the human epidermal growth factor (EGF, Sigma-Aldrich), cells were cultured under serum-free conditions for 24 h followed by the addition of EGF and, optionally, inhibitors of different ABC transporters (Sigma-Aldrich), or DMSO as control for further 12–15 h. HaCaT cells were stained with 2.5 ng/ml of the plasma membrane probe FAST DiO (carbocyanine with diunsaturated $\Delta^{9,12}$-C18 alkyl substituents, Invitrogen) diluted in HEPES-buffered solution (HBS) containing 134 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose, 10 mM HEPES (pH 7.4) and 0.2% (w/v) bovine serum albumin for 20 min at room temperature (RT). After washing with HBS, cells at the edge of larger cell assemblies were examined. A sketch and a detailed explanation of the used setup for FRAP measurements using TIR excitation can be found in a previous publication [12]. For imaging of FAST DiO-stained HaCaT cells, we used the attenuated 488 nm line of an Ar^+ laser (Lasos, Jena, Germany) selected by an AOTF (AA Opto-Electronic, Orsay Cedex, France). A 514 nm beamsplitter (Chroma, Rockingham, VT) allowed for the separation of excitation and emission, and the emission light was filtered by a 514 nm RazorEdge longpass filter (Semrock, Rochester, NY). The 514 nm line was employed for bleaching of a diffraction-limited spot in the plasma membrane of the cells. Images were acquired using an EMCCD camera (iXon DV887, Andor, Belfast, UK) controlled by the TILLvisION software (TILL Photonics, Gräfelfing, Germany). One FRAP cycle consisted of 16 prebleach images, acquired every 20 ms, followed by a 5-ms lasting spot bleaching and immediate acquisition of 20 images in 20-ms intervals. This protocol was 10 times repeated every 10 s. After background correction of the images, the diffusion coefficient was approximated by a convolution-based algorithm as described previously [13].

For FRAP measurements with siRNA-mediated knockdown of ABC transporters in HaCaT cells, we cotransfected HaCaT cells with appropriate siRNA constructs and with a plasmid coding for an RFP-labelled nuclear protein to identify the transfected cells or with the plasmid encoding the nuclear RFP alone for control measurements. Due to its locally restricted cellular expression, the nuclear RFP did not influence the TIR/FRAP measurements and additionally possessed spectral properties that could be sufficiently separated from those of DiO (excitation of the RFP occurred with the 488 nm line of the Ar^+ laser and emission was filtered with a 685/40 nm bandpass filter). Seven hours after transfection, HaCaT cells were starved for 4 h, followed by an incubation of 12–15 h in 10 ng/ml EGF-containing, serum-free cell culture medium prior to FRAP measurements.