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β-1,3/1,4-Glucan Lichenan from *Cetraria islandica* (L.) ACH. induces cellular differentiation of human keratinocytes

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Abstract.

Lichenan (molecular weight 275 kDa, β-D-1,3/1,4-glucopyranose ratio 1:3) from *Cetraria islandica* at a concentration of 100 μg/mL induced terminal cellular differentiation of primary human keratinocytes as demonstrated by immunofluorescence staining using cytokeratin 10 and involucrin as marker proteins. Lichenan-derived oligosaccharides (DP3 to 8), obtained by acid-catalyzed partial hydrolysis of the polymer, did not influence cellular differentiation. Cytokeratin, filaggrin, involucrin, loricrin and transglutaminase gene expression as typical differentiation markers was increased by lichenan in a time-dependent manner. Lichenan upregulated gene cluster which were mostly related to cellular differentiation with focus on MAPK signaling as was shown by Whole Human Genome Microarray. These gene expression data from the array experiments were subsequently confirmed by qPCR for selected genes. For identification of the molecular binding structures of lichenan 1- and 2-D PAGE of keratinocyte protein membrane preparations was performed, followed by blotting with FITC-labeled lichenan and subsequent mass spectrometric identification of the pinpointed proteins. Epidermal growth factor receptor (EGFR) and integrin β4, both proteins being strongly involved in induction of keratinocyte differentiation were identified. In addition, protein disulfide isomerase A3 (PDIA3) showed strong binding to FITC-lichenan, indicating this enzyme to be an intracellular target of the glucan for induction of the cellular differentiation of keratinocytes. As lichenan did not influence the EGFR phosphorylation and the phosphorylation of CREB transcription factor but strongly interacted with cytosolic proteins it is hypothized that the glucan may interact with EGFR and is subsequently internalized into the cell via endosomal uptake, interacting with PDIA3, which again alters TGFβ1 signaling towards keratinocyte differentiation.

1. Introduction

Wounding, especially ulcera are still a big problem in the clinical practice all over the world. For example leg ulcerations are suggested to occur at an incidence of about 1% of the population; in the U.S. chronic wounds affect 3 to 6 million patients with an estimated annual costs of $5 to 10 billion each year [1]. Therefore, the need for intensified development of new lead compounds for effective and evidence-based wound healing strategies is obvious. Active compounds for improved wound healing may stimulate keratinocyte proliferation to generate epidermal cell mass, but can also act on dermal fibroblasts for generation of an improved dermis. Antiinflammatory, antiseptic and

Abbrevations: CLSM, Confocal laser scanning microscopy; CK, Cytokeratin 10; CREB, Transcription factor cAMP response element-binding protein; DP, Degree of polymerization; EGFR, Epidermal growth factor receptor; FITC, Fluorescein isothiocyanate; GPC, Gel permeation chromatography; HaCaT, Human adult low Calcium high Temperature keratinocyte cell line; HP-SEC, High pressure size exclusion chromatography; L, Lichenan from *Cetraria islandica*; MLC, Liquid chromatography; MS, Mass spectrometry; NHEK, Primary normal human epidermal keratinocytes; MW, Molecular weight; PAGE, Polyacrylamide gel electrophoresis; PDI, Protein disulfide isomerase; qPCR, Quantitative real-time polymerase chain reaction; TFA, Trifluoro acetic acid; UC, Untreated control.

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immunomodulating drug properties are reasonable for the successful development of optimized wound healing compounds. No focus has been laid on research for compounds interacting with the keratinocyte differentiation towards the typical barrier cells of the epidermis. From the available drugs interacting with the cell differentiation only retinoids can influence this state of the cell physiology [2] but due to the pronounced toxicity of these compounds the respective use for wound healing is extremely limited. Recently, polysaccharide containing plant extracts have been reported to stimulate keratinocyte differentiation [3] and interestingly these polymers have been applied nowadays in biotechnological manufacture of artificial skin and bone equivalents [4]. Follow up studies have prompted the isolation and characterization of a xyloglucan from *Tropaeolum majus* seeds, which was shown to be a strong inductor of terminal differentiation in skin cells by specific inhibition of the epidermal growth factor receptor (EGFR) on the cell membrane, influencing the intracellular cell signaling towards reduced activation of transcription factor CREB, which again triggers the cell from the proliferative state into the terminal differentiation [5]. As this xyloglucan is characterized by a branched β-1,4-glucan backbone the following study aimed to investigate the pharmacodynamics potential of other β-glucans for differentiation-inducing effects. Interestingly, mixed-linked β-1,3/1,4-glucans seem to induce keratinocyte differentiation via interaction with EGFR and via MAPK signaling, but not by influencing the transcription factor CREB as described for the xyloglucan earlier [6].

Furthermore, β-glucans from plants and algae, serving physiologically as storage and structural polymers have the advantages of originating from sustainable resources. β-Glucans are known to influence immune cells, but also epidermal and dermal cells by interaction with receptors as Dectin-1, Toll-like receptors (TLR-2,4,6), complement receptor 3 (CR3) towards changed cell physiology [7, 8]. Especially immunoactivating properties of β-1,3/1,6 glucans have been investigated intensively, leading to the establishment of these polysaccharides as *Biological Response Modifiers*, used for example in the adjuvcant treatment of cancer to prevent metastasis formation [9, 10]. β-Glucans which are described for improved wound healing interact with immune cells of the skin, cutaneous cells and endothelial cells, leading to changes in cellular proliferation, maturation, migration and differentiation – for review see [8]. Despite the fact that the clinical effects of β-glucans have been documented [11–14] and also innovative galenical formulations for improved and modern topical application has been reported [19–21] detailed understanding of then molecular mechanisms on how these glucans act on the cell physiology is missing in most cases.

Therefore the following study aimed to investigate the influence of a β-1,3/1,4-glucan on human keratinocytes to clearly identify the influence of this polysaccharide on the cell physiology, but also to define the underlying molecular mechanism to understand how these glucans act on protein and gene level on the skin cells. As a model compound for this study, lichenan was used, as this polysaccharide either can be obtained on a commercial basis or can be isolated in a very cost effective and economic way from the lichen *Cetraria islandica* (Iceland moss). Lichenan in this study stands for other mixed linked β-1,3/1,4 glucans (e.g. Avena glucan, Barley glucan etc.) which have similarities to lichenan and differ only concerning the respective mean molecular weights and the ratios of 1,3- to 1,4-linked glucose moieties. These mostly more or less helical 1,3/1,4-linked glucans have strongly to be separated from linear β-glucans as e.g. cellulose-like polysaccharides or β-1,4-linked glucans with side chains as e.g. xyloglucans.

Isolation of the β-glucan lichenan (syn. Lichenin in older literature) can be performed by extraction of the thallus of the lichen, which is a very common ocean lichen species the cold oceans by boiling water. As lichenan is only hot-water soluble coextracted other polysaccharides (e.g. the α-1,3/1,4-glucan isolichenan, cold-water soluble) can ve removed from the extract by repeated freezing and thawing of the aqueous solution [23]. Final purification of lichenan can be performed by precipitation from alkaline solution [23] or by ion exchange chromatography. Especially the use of DEAE-Sephacel anion exchange gel will remove additionally coextracted polyphenols [15, 17]. As the yields of the pure polysaccharide can be > 10%, relate to the lichen starting material, lichenan is the prototype of a useful and economic ingredient for wound healing. Also, the physicochemical gel-forming and the relative insolubility in cold water predestinate this polysaccharide for preparation of gel-like wound dressings, which on one side can stimulate the keratinocyte physiology and on the other side the gel is able to absorb exudates and liquids from the wound.

2. Materials and methods

2.1. General methods of analysis

If not stated otherwise all chemicals were obtained from VWR (Darmstadt, Germany). Lichenan L (Lot 87H0276) was purchased from Sigma-Aldrich (St. Louis, USA).

The following antibodies were used: β-Actin (AC-15), mouse monoclonal (1:4000) (Sigma-Aldrich, St. Louis USA), Alexa Fluor 488 rabbit anti-mouse IgG (1:300) (Life Technologies, Carlsbad, USA), Alexa Fluor 594 goat anti-rabbit IgG (1:300) (Life Technologies, Carlsbad, USA), Cytokeratin 10, rabbit both, monoclonal and polyclonal (dilution: 1:15000, 1:500 respectively) (Abcam, Cambridge, UK), goat Anti-Rabbit IgG HRP (1:10000) (Jackson ImmunoResearch, West Grove, USA), Involucrin (SY5) mouse monoclonal antibody (1:200) (Thermo Scientific, Waltham, USA), Mouse TrueBlot® ULTRA Anti-Mouse Ig HRP (1:1000) (Rockland Immunochemicals, Gilbertsville, USA).

2.2. Analytical characterization of lichenan

Analytical characterization was performed according to the methods already described [15–18]. Lichenan was coupled to fluorescein isothiocyanate (FITC) as presented by [22].

2.3. Enzymatic degradation of lichenan

Enzymatic degradation of lichenan was accomplished by the *endo*-1,3-1,4-β-D-glucanase lichenase (Megazyme, Bray, Ireland). 25 mg of lichenan were dissolved in 1 mL of sodium acetate buffer, pH 4.2 by heating to ~100 °C. After cooling down, 2 μL of lichenase were added to the solution which was subsequently incubated in a thermomixer at 37 °C for 1 h. Lichenase was deactivated by heating to 100 °C for 3 min. The clear supernatant was collected after centrifugation at 14000 × *g* for 5 min and used for subsequent gel-permeation chromatography on a Bio-Gel® P-2 column (BioRad, München, Germany). Chromatographic conditions: column dimensions 1.5 × 87 cm, sample volume 1 mL, mobile phase Aqua Millipore®, flow rate: 0.3 mL/min, fraction size: 2 mL, fraction number: 200.

2.4. Nanoelectrospray quadrupole time-of-flight mass spectrometry (NanoESI-Q-TOF MS)

A NanoESI-Q-TOF1 mass spectrometer (Micromass, Manchester, UK) was used for determination of oligosaccharides generated by enzymatic degradation of the respective polysaccharides. Samples were dissolved in methanol/water/formic acid (49:49:2, v/v/v) and injected by self-pulled nanospray glass capillaries. Ionization by nanoESI was carried out in the positive ion mode using a Z-spray source and following conditions: source temperature 80 °C, desolvation gas N_2 with a flow rate of 75 L/h, capillary voltage potential 1.1 kV and cone voltage 40 V. Data were analyzed by MassLynx™ V4.1, Waters Laboratory Informatics, Milford, USA.