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Carbohydrate Research 343 (2008) 501–511
Carbohydrate RESEARCH

Photoprotective effects of glucomannan isolated from *Candida utilis*

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Received 15 August 2007; received in revised form 8 November 2007; accepted 11 November 2007
Available online 19 November 2007

Abstract—Glucomannans belong to yeast and fungal cell wall polysaccharides with known immunostimulatory and radioprotective effects. However, glucomannan protective effects against pathological consequences of skin exposure to short wavelength solar light, ultraviolet (UV) radiation, are unclear. Herein, a highly branched glucomannan (GM) isolated from the cell wall of *Candida utilis*, a member of the α-(1→6)-D-mannan group, was tested for its photoprotective effects in an in vitro model of UVB-irradiated human keratinocytes and an in vivo model of UV-induced erythema formation in human volunteers. GM suppressed the UVB-induced decrease of keratinocyte viability, which was connected with the suppression of UVB-induced keratinocyte apoptosis. GM reduced UVB-mediated caspase activation together with suppression of DNA fragment release into the cytoplasm. Furthermore, GM suppressed UVB-induced gene expression of pro-inflammatory markers including nuclear factor kappa B, inducible nitric oxide synthase, interleukins 8 and 1, together with suppression of prostaglandin E2 and interleukin 1α protein release. In vivo, GM decreased UV-induced skin erythema formation, which was correlated with a decrease of phosholipase A_2 activity within the stratum corneum. It could be concluded that GM isolated from *C. utilis* possesses significant photoprotective effects on human keratinocytes in vitro as well as in vivo.

Keywords: Glucomannan; *Candida utilis*; HaCaT keratinocytes; UV-protection; Polysaccharide; Apoptosis

1. Introduction

It is well recognized that skin exposure to solar radiation has detrimental consequences, both acute and chronic. In particular, the short wavelength part of solar light, ultraviolet (UV) radiation, contributes significantly to undesirable effects, which could lead to the development of cutaneous malignancies.[1–3] The impact of cutaneous malignancies is significant since melanoma and non-melanoma skin cancer are the most abundant carcinomas in western countries.[1,4] Therefore, development and improvement of skin protection strategies against solar radiation is a critical issue.

Different mechanisms are suggested to contribute to the adverse effects of UV radiation on the skin. One of the most significant mechanisms is UV-induced suppression of immune functions connected with massive cutaneous cell death. Decrease of cutaneous cell viability is related to UV-induced cell damage due to the formation of free radicals that destruct cellular structures including lipids, nucleic acids, and proteins.[1,2] Physiological functions associated with UV-induced impairment of skin are directly associated with the inflammatory response provoked by the damaged keratinocytes, which leads to the release of a wide range of inflammatory mediators. Cytokines released during this early phase of UV-induced skin reaction are considered to be impor-

Abbreviations: αMSH, alpha melanocyte stimulating hormone; CR3, complement receptor 3; GM, glucomannan; iNOS, inducible nitric oxide synthase; IL-1α, interleukin 1 alpha; MR, mannose receptor; NO, nitric oxide; NFκB, nuclear factor κB; PAMPs, pathogen-associated molecular patterns; PLA_2, phospholipase A_2; PGE_2, prostaglandin E_2; TLR, toll-like receptor; UVB, ultraviolet light B

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doi:10.1016/j.carres.2007.11.010

tant mediators of the consequent immune suppression in skin.[5] Therefore, the prevention of UV-induced keratinocyte injury could significantly reduce the detrimental effect of UV radiation on skin.[1,2]

Increased resistance of cells to different types of noxious stimuli is connected with an increase in the activity of various intracellular protective and repairing mechanisms, which resemble adaptation to cellular stress.[6,7] Activation of stress-induced signaling pathways by non-specific stressors, such as heat or radiation leads to the activation of cytoprotective responses.[8] Similar cell responses could be induced through the activation of specific cell surface receptors including receptors for microbial molecular patterns (or pathogen-associated molecular patterns, PAMPs) comprising lipids, carbohydrates, proteins, and nucleic acids because their molecular structure is distinct from those expressed on the surface of mammalian cells.[9–12] Polysaccharides that are part of the cell wall of yeasts and fungi are among the molecular structures recognized by these receptors.[9–12] Therefore, biologically active polysaccharides with the ability to active cellular responses in skin could be skin-protective agents.

Yeast and fungal polysaccharides consist of glucose and mannose units joined together by glycosidic linkages via different positions and in different ratios. The biological activity of purified glucans and glucomannans, including decrease of infectious complications and inhibition of tumor growth, is known to depend on their structure. Parameters such as primary structure, degree of branching, molecular weight, solubility, solution conformation, and ionic charge were suggested to play a role in determining the biological activity of these molecules.[13,14] In a previous study, we observed strong immunostimulatory effects of two structurally different polysaccharides, schizophyllan, and carboxymethylglucan, which were isolated from *Schizophyllum communae* and *Saccharomyces cerevisiae* cell walls, respectively.[15] However, the relationships between the structure of glucans and glucomannans and their stimulatory activities still remain unclear. Glucomannan (GM) isolated from *Candida utilis* consists of the α-(1→6)-D-mannopyranosyl backbone carrying mannooligosaccharidic side chains (1–5 units) containing α-(1→2) linkages, where some of the side chains are terminated with non-reducing D-glucopyranosyl residues.[16] Generally, the mass of this polysaccharide varies between 30 and 70 kDa and the mannose/glucose ratio is 2–3:1 (Fig. 1).

Glucans and glucomannans exhibit various biological activities, which are mediated by interaction with cell surface receptors. GM is recognized by several types of receptors including complement receptor 3 (CR3), the mannose receptor (MR), toll-like receptors (TLRs), and other lectin receptors, which are widely expressed on leukocytes and mediate cellular response to different types of PAMPs.[10] However, mannan/glucan binding

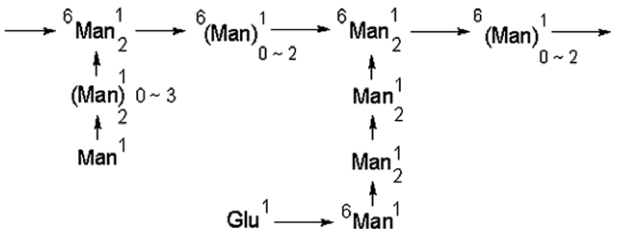

Figure 1. Structure of GM isolated from *Candida utilis*, which consists of an α-(1→6)-D-mannopyranosyl backbone carrying mannooligosaccharidic side chains (1–5 units) composed of α-(1→2) linkages, where some of the side chains are terminated with non-reducing D-glucopyranosyl residues.

sites have also been described on other cell types including fibroblasts.[10] and different epithelial cells.[17,18] These glucan/mannan receptors could also be assumed to be present on keratinocytes. Indeed, keratinocytes were already shown to express lectin receptors and toll-like receptor 4, which were suggested to be crucial for the recognition of O-linked mannosyl polymers such as glucomannans.[7,19,20]

Given the above premises, it could be hypothesized that GM acts as a photoprotective agent and prevents UVB-induced damage of cutaneous cells. The photoprotective properties of the GM isolated from *C. utilis* were evaluated in vitro on UVB-irradiated primary human keratinocytes and the immortalized keratinocyte cell line HaCaT. Furthermore, the photoprotective effects of the GM were confirmed in vivo by the measurement of UV-induced erythema formation in human volunteers. Parameters of the skin inflammatory response (phospholipase A_2 activity) were determined in stripped layers of stratum corneum. The data obtained show significant GM protective effects against UVB-induced death of human keratinocytes and suggest GM as a potent photoprotective agent.

2. Results

2.1. UVB exposure induced inflammatory response and apoptosis in keratinocytes

To explore the photoprotective properties of GM, model of primary human keratinocytes and human keratinocyte cell line HaCaT irradiated by UVB light was employed. Doses of UVB radiation (10 and 20 mJ/cm^2) and concentrations of GM (50 and 500, 1000 μg/mL) were selected based on the preliminary results (data not shown). Upon irradiation with 10 mJ/cm^2 UVB, the viability of both keratinocyte cell cultures decreased significantly after 24 h. Thus, the dose 10 mJ/cm^2 of UVB was selected for cell viability evaluation and for the induction of the cell inflammatory response. A higher dose of UVB (20 mJ/cm^2) was selected for the

examination of GM-dependent modulation of apoptotic processes due to significant induction of apoptosis by this dose up to 5 h after the treatment.

2.2. GM prevented the UVB-induced decrease of cell viability in primary keratinocytes

GM did not significantly modulate cell viability of cells not treated with UVB (Fig. 2a). However, in agreement with our hypothesis, GM at concentrations 50 and 500 μg/mL revealed a protective effect on the viability of irradiated keratinocytes (Fig. 2b). Interestingly, comparison of GM isolated from *C. utilis* and GM isolated by the same method from *S. cerevisiae* showed the higher protective effect on keratinocyte viability of the former (data not shown). This confirmed our selection of GM isolated from *C. utilis* for further characterization of GM photoprotective effects.

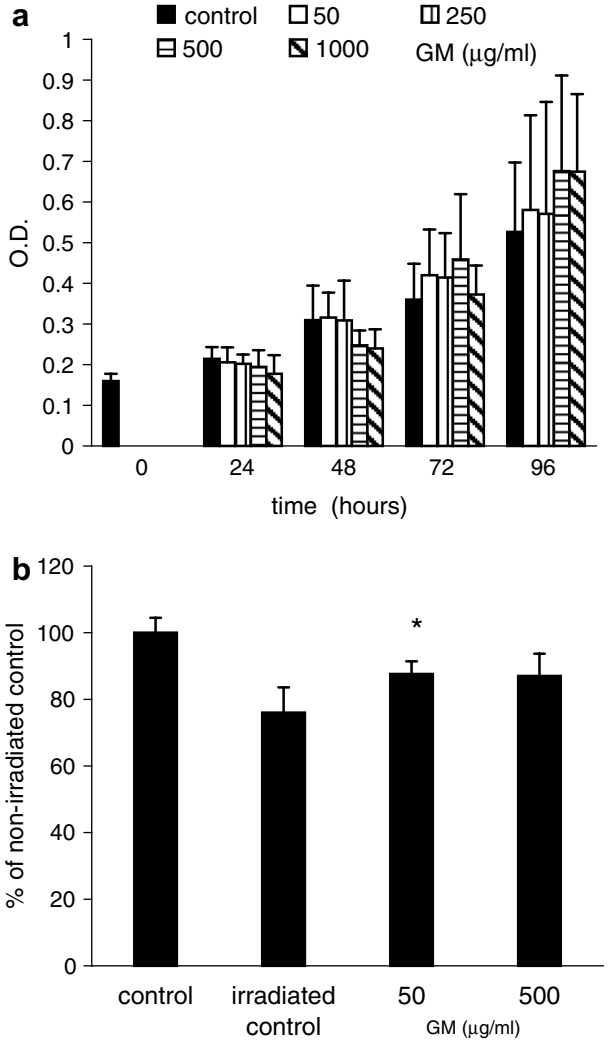

Figure 2. GM did not modulate the viability of intact human primary keratinocytes but did reduce UVB-induced decrease of keratinocyte viability. (a) Human primary keratinocytes were treated by GM (50, 250, 500, and 1000 μg/mL) for 24, 48, 72, and 96 h and the cell viability was determined by the XTT test. (b) Human primary keratinocytes were irradiated by UVB (10 mJ/cm^2) and treated with GM (50 and 500 μg/mL) immediately after irradiation. Cell viability was determined 24 h after the UVB exposure by the XTT test. Non-irradiated keratinocytes served as the control. Three determinations were performed and data represent mean ± standard error of mean.

2.3. GM prevented the UVB-induced apoptosis of primary keratinocytes

GM-mediated alternation of keratinocyte apoptotic cell death induced by UVB was evaluated to characterize the protective effects of GM on the viability of UVB-irradiated keratinocytes. The apoptotic process was characterized based on the determination of caspase 3 and pan-caspase activity, as well as the formation of low molecular weight DNA fragments (mono- and oligo-nucleosomes) in the cytoplasm of keratinocytes irradiated by UVB (20 mJ/cm^2) 5 h after the treatment. GM significantly suppressed the activation of caspase 3 and the formation of low molecular weight DNA fragments in the keratinocyte cytoplasm (Fig. 3a and b). Similarly, UVB-induced pan-caspase activation was notably suppressed by GM treatment (Fig. 4). GM treatment alone did not induce any detectable increase of these apoptotic markers (data not shown).

2.4. GM decreased the formation of thymidine dimers in UVB-irradiated primary keratinocytes

The formation of thymidine dimers in UVB (10 mJ/cm^2) irradiated primo-cultures of keratinocytes was evaluated since thymidine dimers are the most common cause of development of DNA lesions after the exposure of cells to UVB irradiation. Interestingly, treatment by GM decreased, in a dose dependent manner, the abundance of thymidine dimers in the irradiated keratinocytes 3 and 6 h after irradiation (Fig. 5a and b).

2.5. GM prevented an increase of pro-inflammatory markers in UVB-irradiated HaCaT

GM significantly down-regulated gene expression of nuclear factor κB, pro-inflammatory interleukin-1α (IL-1α), interleukin-8 (IL-8), and inducible nitric oxide synthase (iNOS) in UVB-irradiated (10 mJ/cm^2) HaCaT (Table 1). Moreover, GM-dependent down regulation of IL-1α and prostaglandin E_2 (PGE_2) release from UVB-irradiated (10 mJ/cm^2) HaCaT was observed already 24 h after cell irradiation (data not shown). This effect was more profound 48 h after the treatment (Fig. 6a and b). GM application alone did not induce a significant release of IL-1α and PGE_2 from untreated keratinocytes (Fig. 6a and b).

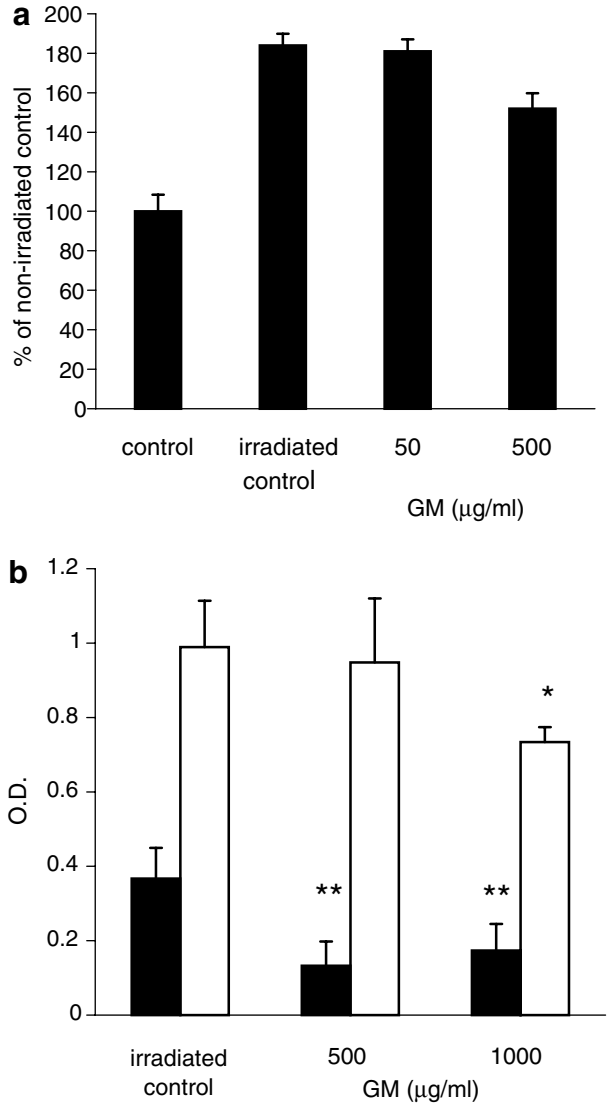

Figure 3. Reduction of (a) the caspase 3 activity and (b) low molecular weight DNA fragments (mono- and oligo-nucleosomes) in the cytoplasm of UVB-irradiated human primary keratinocytes by GM. Human primary keratinocytes were irradiated by UVB (20 mJ/cm^2) and treated with GM (50, 500 and 1000 μg/mL) immediately after irradiation. (a) Caspase-3 activity was determined in cell lysate 5 h after UVB exposure. Non-irradiated keratinocytes served as the control. Three determinations were averaged and data represent mean ± standard error of mean. (b) Mono-and oligo-nucleosomes were determined 5 h (black columns) and 24 h (white columns) after the treatment. Non-irradiated keratinocytes served as the control. Four determinations were performed and data represent mean ± standard error of mean.

2.6. GM induced α-melanocyte stimulating hormone (αMSH) in UVB-irradiated primary keratinocytes

The release of αMSH by keratinocytes was evaluated by considering αMSH to be an autocrine mediator with a potent effect on cell protective mechanisms when released by keratinocytes in response to injurious stimuli.[21,22] Consistently with the observed GM photoprotective effects, GM significantly potentiated the release of αMSH by UVB-irradiated primary keratinocytes (120–169% of irradiated control).

2.7. GM protected skin against UV-induced erythema formation in vivo

The photoprotective effect of GM against UV-induced erythema formation in human volunteers was studied. The application of GM (0.5% in emulsion) on skin visibly decreased UV-induced erythema in four of five volunteers (Fig. 7). The effects of GM were quantified by the evaluation of erythema indexes, which were significantly decreased [25.32 (±2.35) compared to 28.78 (±2.85), $p < 0.05$] and a^* values of CIE $L^*a^*b^*$ color space [5.1 (±0.41) compared to 5.66 (±0.57), $p < 0.05$] in comparison to the control irradiated sites.

2.8. GM lowered phospholipase A2 (PLA_2) activities in the stripped stratum corneum

To characterize the protective effect of GM on UV-irradiated skin, PLA_2 activity in the stratum corneum stripped from irradiated sites was evaluated. GM-treated sites revealed lower PLA_2 activity compared to control sites [160 (±27.5) vs 223.7 (±69)] suggesting a mechanism for PLA_2 to be involved in the protective effect of GM in a model of UV-induced erythema in vivo.

3. Discussion

The aim of the study was to evaluate the photoprotective effects of biotechnologically prepared GM against UVB adverse effects on human keratinocytes using in vitro as well as in vivo approaches. GM isolated from *C. utilis* and from *S. cerevisiae* possessed the distinct ability to prevent cytotoxic effects of UVB radiation on human keratinocytes, with the former showing higher potential. This effect was shown to be connected with the reduction of UVB-induced keratinocyte apoptosis. Furthermore, GM isolated from *C. utilis* significantly decreased expression and release of selected pro-inflammatory markers and mediators. In contrast, GM potentiated the release of αMSH, a potent mediator of skin protection. Finally, the protective effect of GM against UVB-skin damage was confirmed by a reduction in UV-induced erythema formation on the skin of human volunteers. These effects correlated with the GM-mediated reduction of UV-induced increase of PLA_2 in stratum corneum. These data suggest potent photoprotective effect of GM against UVB irradiation.

Here, UVB light was used as a model for the evaluation of the adverse effects of sun radiation on skin. From

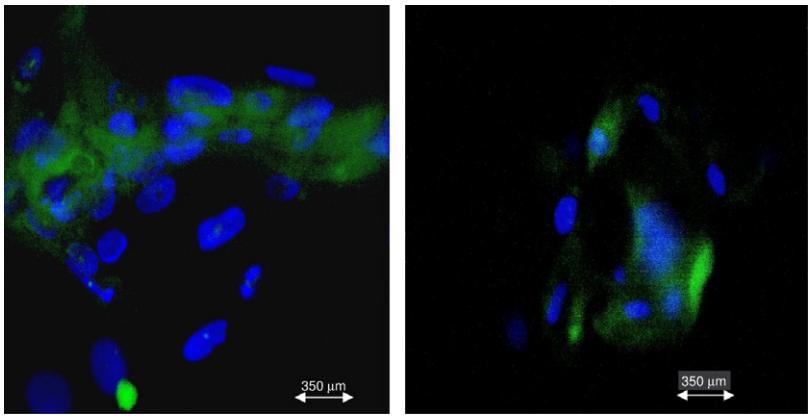

Figure 4. GM reduced pan-caspase activation in UVB-irradiated human primary keratinocytes as determined by fluorescence microscopy. Human primary keratinocytes were irradiated by UVB (20 mJ/cm^2) and treated with GM (500 µg/mL) immediately after irradiation. Pan-caspase activity in the cytoplasm was determined by CaspACE™ FITC-VAD-FMK. An in situ marker (Promega, Vienna, Austria) was added 24 h after the treatment. Magnification: 400×, used microscope Nikon Eclipse E400 (Tokyo, Japan) visualization software Lucia v. 4.82; blue color-lowest nuclei labeled using Hoechst. Left side: irradiated keratinocytes; right side: irradiated keratinocytes treated with GM (500 µg/mL). Four determinations were performed and typical figures were presented.

the spectrum of sunlight, UVB light has the lowest potential to penetrate skin, but it is considered to be the main mediator of cutaneous damage and inflammation leading to the formation of skin carcinomas and to the alteration of cutaneous immune responses.[23] Adverse effects of UVB on cutaneous cells could be prevented by the activation of cellular mechanisms protecting cells against the damaging effects of toxic agents such as UVB irradiation. The cytoprotective mechanisms could be triggered by various factors including substances recognized by receptors for microbial molecular patterns or PAMPs. Therefore, GM could be suggested as a substance with potential to activate cellular mechanisms leading to an increase of cutaneous cell resistance to UVB irradiation.

GM revealed a protective effect on the viability of UVB-irradiated keratinocytes. This observation is in agreement with other authors who reported anti-mutagenic, anti-genotoxic, and anti-cancerogenic properties of GM in various model systems.[24,25] Furthermore, direct photoprotective effects of polysaccharides were reported for carboxymethyl glucan, which increased resistance of corneal epithelial cells to UVB- and hydrogen peroxide-induced cell death.[11]

The protective effect of GM against UVB-induced cell death was directly connected with reduced apoptosis of keratinocytes. GM significantly decreased the markers of early stages of apoptotic process: the activation of caspase 3, the activation of pan-caspase, and the release of mono- and oligo-nucleosomes into the keratinocyte cytoplasm.[26–28] Caspases, a family of cysteine proteases, are the common executors of apoptosis and are induced by various stimuli.[28] GM-mediated suppression of UVB-induced caspase activation could be connected with the suppression of a poly(ADP-ribosyl)polymerase activation, as documented by the significantly lower levels of low molecular weight DNA fragments detected in the cytoplasm of GM-treated keratinocytes. Therefore, it could be speculated that GM protects cutaneous cells from UVB-induced apoptosis by the suppression of caspase activation and subsequent hindering of DNA fragmentation. Similar to keratinocytes, GM treatment of the UVB-irradiated Jurkat T lymphocyte cell line decreased the activation of pan-caspase (data not shown).

GM was shown to significantly decrease detectable thymidine dimers in UVB-irradiated keratinocytes. The abundance of thymidine dimers was analyzed as it is considered to be the most common cause of development of DNA lesions after the exposure of cells to UVB irradiation.[29,30] Although a large fraction of these DNA lesions are repaired enzymatically by nucleotide excision repair, the amount of unrepaired lesions is not negligible and could result in UVB-induced mutagenesis.[29] The observed GM-mediated reduction of thymidine dimers suggests a potentiation of the DNA damage repair mechanisms in keratinocytes. This indicates another mechanism of GM photoprotective effect on UVB-irradiated human keratinocytes. The faster elimination of thymidine dimers in presence of GM provides other evidence for reparative effects evoked by GM.

Upon UV irradiation, cutaneous cells including keratinocytes produce a wide range of inflammatory mediators.[23,31] Interestingly, microarray analyses showed that GM treatment significantly suppressed UVB-induced NFκB gene expression in keratinocytes. This correlates with the observed GM-mediated down regulation of gene expression of the tagged pro-inflammatory markers IL-1α, IL-8, and iNOS. Furthermore, GM-dependent down regulation of IL-1α and PGE_2 gene expression was confirmed by an observed decrease of

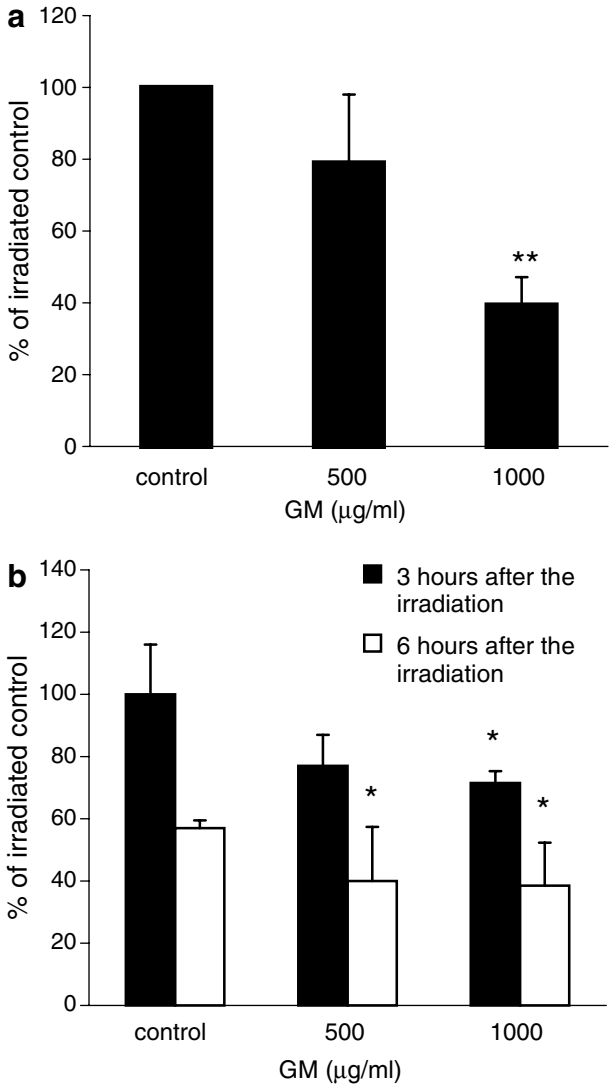

Figure 5. GM decreased the formation of thymidine dimers in UVB-irradiated primary keratinocytes. Human primary keratinocytes were irradiated by UVB (10 mJ/cm^2) and treated with GM (500 and 1000 μg/mL) immediately after irradiation. Levels of thymidine dimers in primary keratinocytes (a) and HaCaT (b) were determined in cell culture lysate after 3 h (black columns) and 6 h (white columns) after the treatment. Irradiated untreated keratinocytes served as the control. Four (the 3-h treatment) and two (the 6-h treatment) determinations were performed and data represent mean ± standard error of mean.

IL-1α and PGE_2 protein release from UVB-irradiated keratinocytes. IL-1α and IL-8 are potent pro-inflammatory mediators and together with the increased production of NO, catalyzed by iNOS, contribute to acute inflammatory response to UV irradiation.[31,32] Similar to the observed effects of GM, polysaccharides isolated from *Grifola frondosa* were reported to decrease expression and release of matrix metalloproteinase 1, which is another significant mediator contributing to the development of inflammatory reaction in UV-irradiated skin.[9]

Table 1. Gene expression profiling of GM effects on UVB-irradiated human keratinocyte cell line HaCaT[a]

Gene	Percentage of change against irradiated control (%)
PTGS2	−89
FGF2	−85
TP53	−89.50
IL-8	−84
NFκB	−84
IL-1	−83.70
iNOS	−82.50
TLR4	−82
MMP3	−82
FGF9	−80.40
ARNT	−80

[a] Human primary keratinocytes were irradiated by UVB (10 mJ/cm^2) and treated with GM (500 μg/mL) immediately after irradiation. GM-mediated modification of gene expression profiles was evaluated by microarray assay 24 h after treatment. Up-regulation is indicated by +, down regulation by −, and intensity is expressed as % modification as compared to irradiated control without GM treatment. Gene abbreviations: PTGS2- cyclooxygenase 2; FGF- fibroblast growth factor, TP53- apoptotic protein p53; IL—interleukin; NFκB—nuclear factor κB; iNOS—inducible nitric oxide synthase, TLR—toll-like receptor, MMP3-matrix metalloprotease, ARNT—aryl hydrocarbon nuclear translocator.

Together with the decrease of pro-inflammatory mediator expression, GM augmented the release of αMSH by irradiated keratinocytes.[33] αMSH is tridecapeptide derived from propiomelanocortin and is involved in a variety of biological processes, such as pigmentation or extracellular matrix composition.[34,35] αMSH possesses significant anti-inflammatory activities mediated through the activation of αMSH receptors resulting in a decrease of inflammatory mediator production or leukocyte migration.[21,22,36] Therefore, it could be suggested that GM-mediated increase of αMSH formation may trigger keratinocytes in an autocrine fashion, which reduces inflammatory response after UVB irradiation.

To confirm the photoprotective effects of GM observed in vitro, GM modulation of UVB-induced erythema was evaluated in vivo on the skin of healthy human volunteers. In agreement with in vitro data, the application of GM on the skin significantly decreased UV-induced erythema formation. Application of GM considerably attenuated the activity of PLA_2, a pro-inflammatory enzyme activating leukotriene synthesis[37] in the stripped stratum corneum. Interestingly, the activity of β-glucocerebrosidase, an enzyme contributing to the repair of UVB-damaged skin,[38] was slightly increased by GM in comparison to the untreated sites. This suggests that GM improves a skin barrier function impaired by UV exposure (unpublished data). Another possible explanation for the reduced erythema formation could be connected with the ability of GM to down regulate UVB-induced iNOS expression in vitro. The reduction of iNOS expression and NO overproduction

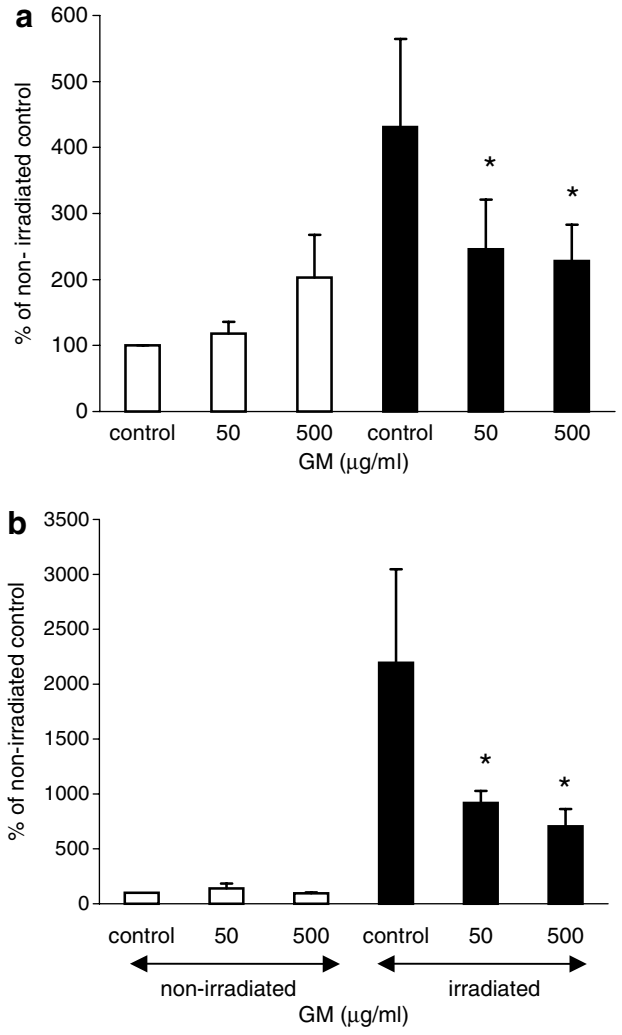

Figure 6. GM reduced the release of IL-1α and PGE_2 by UVB-irradiated human keratinocytes HaCaT into the culture media. Human primary keratinocytes were irradiated by UVB (10 mJ/cm^2) and treated with GM (50 and 500 μg/mL) immediately after irradiation. Levels of (a) IL-1α and (b) PGE_2 were determined in cell culture media 48 h after the treatment (black columns). Non-irradiated keratinocytes treated only by GM for 48 h served as the control (white columns). Four determinations were performed and data represent mean ± standard error of mean.

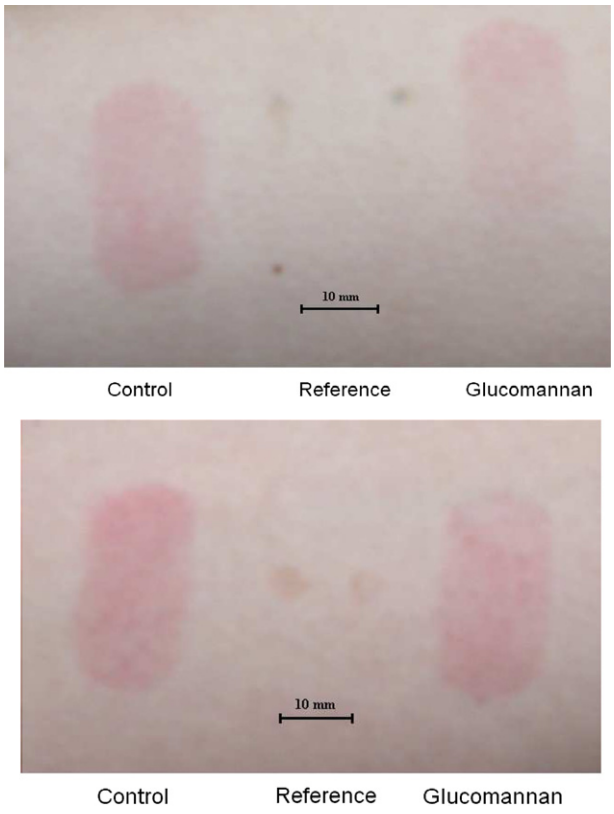

Figure 7. GM-reduced skin erythema induced by UVA/B (1.25 MED). Typical results of erythema skin formation shown on 2 volunteers. Experiments were performed as described in Section 4.

could reduce pathological vasodilatation coupled with UVB induced inflammation.[23,32,39] Moreover, GM inhibition of PGE_2 release that was observed in vitro could significantly reduce the skin inflammatory response to UVB due to a reduction of histamine release.[31,40] Based on our data we could speculate that the GM effect on keratinocytes may evoke an adaptive cell response and may contribute to the activation of reparative processes leading to improved cell viability after the UVB exposure.[6,7]

In conclusion, GM isolated from *C. utilis* was shown to be a potent photoprotective compound preventing harmful effects of UVB exposure on human keratinocytes and skin. GM could therefore be suggested as a protective agent in cosmetic products, which prevents detrimental effects of solar radiation on skin.

4. Experimental

4.1. Human subjects

The present study has been approved by the CPN, s.r.o. Institutional Review Board, and the Declaration of Helsinki protocols were followed. Patients gave their written, informed consent.

4.2. Glucomannan

Glucomannan was isolated from *C. utilis* by sodium hydroxide extraction (2% NaOH, 25 min at 95 °C). HCl was used to adjust pH to 5.5 and the supernatant was clarified by centrifugation. Glucomannan was precipitated by the addition of isopropanol. The precipitate was dissolved in water (40 °C) and filtered several times through paper and active charcoal filters. Finally, glucomannan was precipitated from an aqueous solution with isopropanol, the precipitate was dissolved in absolute isopropanol and dried under vacuum. GM had a molar

mass between 60 and 70 kDa and a narrow molecular distribution (polydispersity index between 1.15 and 1.24) as determined by SEC/MALLS. The ratio of mannan to glucan was 2.73:1 in GM isolated from *C. utilis*. GM isolated from *S. cerevisiae* had a ratio of 30–53 mannans per glucan.

4.3. Cell cultures

The spontaneously immortalized human keratinocytes cell line HaCaT (kind gift of Prof. Dr. N. Fusenig[41]) was grown in DMEM supplemented with 10% of fetal bovine serum (Gibco, Carlsbad, California, USA), glutamine (0,25 mg/mL, Gibco), and gentamicin (50 μg/mL, Gibco) at 37 °C.

Primary normal human epidermal keratinocytes were isolated from the skin removed during cosmetic plastic surgery. Keratinocyte cultures were prepared according to the method of Rheinwald and Green.[42] The skin was minced and trypsinized (0.25% trypsin + 0.02% EDTA) at 37 °C for 16–18 h and keratinocytes were grown in 75 cm^2 culture flasks with mitomycin-treated 3T3 fibroblasts used as a feeder. Keratinocytes were grown in cell culture media DMEM (Sigma–Aldrich, Vienna, Austria), HAM-F-12 (Sigma–Aldrich, Vienna, Austria), newborn calf serum (10%, Gibco), hydrocortisone, adenine, choleratoxin, EGF, insulin, amphotericin B, penicillin, streptomycin, apotransferrin, and 3,3,5-triiodo-L-thyronine (Sigma–Aldrich, Vienna, Austria). The medium was changed every 2 days. Keratinocytes were cultivated until confluence was reached and passaged again at a density $1 \times 10^4/cm^2$. After three passages keratinocytes were seeded at a concentration $1.55 \times 10^4/cm^2$. Keratinocytes were cultivated overnight before the experimental treatment.

4.4. UVB irradiation and GM treatment in vitro

A 1000 W xenon arc solar UV-simulator (Oriel Instruments, Stratford, CT, USA) equipped with a dichroic mirror and a 300 ± 5 nm interference filter was used for cell irradiation in vitro. The UVB output power and radiation doses were determined by using photometer/radiometer PMA 2100 (Solar Light Co., Glenside, PA, USA). For the evaluation of cell viability and inflammatory response, a 10 mJ/cm^2 dose was applied, whereas a 20 mJ/cm^2 dose was used for the cell apoptosis evaluations. Before UVB treatment, the cell culture media were replaced by phosphate buffer saline (pH 7.4, PBS). Immediately after the UVB irradiation, PBS was replaced by cell culture media containing GM. The RNA was isolated by Trizol® Reagent (Invitrogen, Carlsbad, California, USA) 24 h after UVB treatment and stored at −80 °C in DEPC water. The cell culture media were collected 24 and 48 h after UVB treatment and stored at −20 °C until analysis. Cells were harvested and lysed in different buffers according to the determined apoptotic markers 5 h after UVB treatment.

4.5. XTT cell viability assay

Cell viability was determined by the commercial XTT assay (RocheApplied Science, Meylan, France). Cells were incubated with medium containing 33% (v/v) XTT solution for 2 h. An absorbance of 150 μL media was determined by a Versamax microplate reader (Molecular Devices, Union City, California, USA) at a wavelength 450 nm.

4.6. Caspase activity assay

Cells were lysed in 200 μL of a lysis buffer containing 50 mM HEPES (pH 7.4), 0.2% CHAPS, 5 mM DTT, and 0.2 μM aprotinin for 20 min on ice and sonicated 3 times for 15 s. Samples were centrifuged (15,000*g*/10 min/4 °C), and the protein concentration in the supernatant was determined and adjusted to same concentration in all samples. Samples were incubated with assay buffer (20 mM HEPES with pH 7.4, 0.1% CHAPS, 5 mM DTT, and 2 mM EDTA) containing 10 mM caspase substrate (Ac-DEVD-AMC, Sigma–Aldrich, Vienna, Austria) and incubated for 2 h at 37 °C. The fluorescence of the substrate was quantified by an Infinite M200 (Tecan, Männedorf, Switzerland) fluorescence reader with excitation at 380 nm and emission at 460 nm. Pan-caspase activation was detected using the commercial In Situ marker CaspACE™ FITC-VAD-FMK (Promega, Vienna, Austria) kit and observed under fluorescent microscope Nikon E400 (Tokyo, Japan).

4.7. cDNA array

cDNA synthesis and labeling were performed by RevertAid H Minus MuLV-Reverse Transcriptase (Fermentas, Burlington, Ontario, Canada) and Biotin-11-dUTP (Fermentas). Hybridization was performed on the cDNA array system (Clondiag chip technologies GmbH, Jena, Germany). The amount of cDNA in each spot was detected by enzyme reaction of HRP using substrate True Blue (KPL, Gaithesburg, Maryland, USA). The developed color was determined by Reader ATR01 (Clondiag). IconoClust software (Clondiag) was used for spot proceeding and β-actin, GAPD, and histone H3 markers were used as housekeeping markers for the following equalization of the spot intensities.

4.8. Cytokine and DNA fragment ELISAs

PGE_2 and IL-1α in cell culture media were determined in duplicate by commercial ELISA kits (KGE004 from RnD Systems Europe, Ltd, Abington, UK; and BMS 243/2MST from Bender MedSystems, Vienna, Austria,

respectively) according to the manufacturer manuals. Low molecular weight DNA fragments were determined in keratinocyte lysate by a Death Detection Kit following manufacturer instructions (Roche Applied Science, Meylan, France). Two different optimal dilutions were used to perform the assay: 1:40 (vol/vol) for 5 h and 1:67 (v/v) for 24 h intervals. Release of αMSH into the culture medium was determined by commercial αMSH EIA (Phoenix Pharmaceuticals, Inc., Karlsruhe, Germany).

4.9. Detection of thymidine dimers

DNA isolation from cells was performed according to a modified method described by Roza et al.[43] Primary keratinocytes were irradiated by UVB (10 mJ/cm^2), immediately treated with GM (500 and 1000 μg/mL), and incubated for 3 and 6 h before their lysis. Cells were lysed in 200 μL of a buffer containing 10 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5% SDS, and Proteinase K (200 μg/mL). After a 2 h incubation at 55 °C, DNA was purified by phenol extraction, RNase H digestion, chloroform/isoamylalcohol (24:1) extraction and ethanol precipitation. The concentration and the purity of DNA was assessed spectroscopically (UV–vis spectrophotometr UV-2401PC). Further, 50 ng of DNA was absorbed to each well of poly-L-lysine precoated plates (BD Biosciences, San Jose, CA, USA) by incubation at 4 °C overnight. Plates were washed (0.05% vol/vol Tween in PBS), blocked by BSA in PBS (1% wt/vol) for 1 h at room temperature. The primary antibody (anti-thymidine dimer KTM53) diluted 1:10,000 in PBS (Kamiya Biomedicals, Seattle, WA, USA) was added and the plates were incubated on orbital shaker (200 rpm) for 2 h at room temperature. After washing wells 3 times in wash buffer, biotinylated goat-anti-mouse IgG (Dako, Glostrup, Denmark) diluted 1:1000 in blocking buffer was added and incubated on orbital shaker for 1 h at room temperature. Next, plates were washed 3 times by wash buffer and streptavidin-poly HRP (Pierce, Rockford, IL, USA) diluted 1:10,000 in PBS with BSA (0.1% vol/vol) added and incubated on an orbital shaker for 30 min at room temperature. Plates were washed 3 times, TMB substrate (Sigma–Aldrich, Vienna, Austria) was added and the absorbance was read at 450 nm. Results were expressed as percentages of arbitrary absorbance units (AU) minus AU of the wells coated by DNA isolated from non-irradiated controls.

4.10. Skin erythema formation and determination of enzymatic activity in vivo

Seven female and male Caucasian volunteers with healthy skin (Fitzpatrick types II and III), aged 24–39 years, and in a good health were selected after a study approval by the ethics committee. Each person signed an informed consent form; five completed the study. There were three fields, 4 × 3 cm, marked on the volar forearms of human volunteers. An emulsion with 0.5% *C. utilis* GM was applied 3 times a day to the test sites over a 7-day period, as well as a control emulsion without any active material to the control sites. The reference sites were left untreated. For the last three applications on the last day, the emulsions were replaced by a simple carbomer gel with or without 0.5% GM to avoid possible adverse skin reactions to some ingredient due to the UV irradiation. None of the preparations contained preservative and only freshly defrosted ones were used for the treatment every day.

A 1000 W xenon arc solar UV-simulator (Oriel Instruments, Stratford, CT, USA) equipped with a dichroic mirror and a combination of UG5 (1 mm) and WG280 (2 mm) Schott filters giving a COLIPA compliant UV-spectrum was used for the irradiation of the test skin sites. The minimal UVA/UVB erythema doses for all volunteers were determined 2–4 days before testing individually. Skin redness before and 24 h after irradiation was measured using a reflectance spectrophotometer Spectrocam 75 RE (Spectrostar). The reflectance values within the range 380–750 nm were recorded, both a^* values of $L^*a^*b^*$ color space and erythema indexes according Wagner et al.[44] were calculated. On each test site, five determinations were performed and the mean of five values was calculated. Skin redness changes were expressed as the differences between the initial and final a^* and erythema index values measured in the test, control, and adjacent untreated reference skin sites.

After the pre-treatment period, the skin sites were cleansed gently and allowed to dry. The initial a^* and erythema index values were measured and areas 3 × 1.2 cm located on the two pre-treated sites were irradiated with 1.25 MED UVA/UVB. Then, the treatment with GM and control gels continued three times during the following day. The skin was photographed (Coolpix 4500, Nikon, Japan) 24 h after irradiation, the redness was determined spectrophotometrically, and two groups of 11 strips (D-Squame, Cuderm Corp., Dallas, TX, USA) were taken from each skin site. These strips were immersed in 100 mM TRIS/HCl buffer, pH 7.5, containing 0.5% Triton X-100 for 1 h at room temperature. The extracts were collected and used for the enzyme activity determinations. β-Glucocerebrosidase activities were evaluated by fluorescence measurement of 4-methylumbelliferone released from 4-methylumbelliferyl β-D-glucopyranoside at 37 °C (Shimadzu RF-5301PC spectrofluorophotometer, excitation at 358 nm, emission at 448 nm). The PLA_2 activities were estimated by the determination of fluorescent pyrene derivatives released from 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphoglycerol at 37 °C (excitation at 344 nm, emission at 398 nm).

4.11. Statistical analysis

All experiments were carried out with 3–4 replicates in vitro, and with 5 replicates for in vivo observations. The results are reported as a mean ± standard error of mean (SEM) in the figures and mean ± (SEM) in the text. Statistical significance was analyzed by Mann and Whitney distribution-free-U test and considered significant at $p < 0.05$ (*), eventually $p < 0.01$ (**).

Conflict of interest

V.V. has a financial interest in CPN, Dolni Dobrouc, the company providing the tested glucomannan. E.R., S.P., V.H., S.J. are employees of CPN, Dolni Dobrouc. The research of I.P. and L.K. was partially supported by CPN, Dolni Dobrouc, Czech Republic.

Acknowledgments

The authors wish to thank S. Moravcikova for technical assistance with protein and enzymatic assays, M. Magdolenova for technical assistance with erythema measurements, and L. Balonova and A. Franzova for general technical assistance.

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