Unprecedented skin regeneration of *euglena gracilis*-derived extracellular microvesicles fabricated through microalgae extrusion

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Abstract

Background: β-glucans from different sources have various functions depending on their molecular structures. Especially *euglena gracilis* containing β-1,3-glucan as a form of paramylon could contribute to epithelial migration and proliferation without any toxic effects in skin cells.

Methods: Pressurizing *E. gracilis* through a permeable membrane enables fabrication of *E. gracilis*-derived extracellular microvesicles (EGMVs). Aniline blue assay and BCA assay were used to measure the concentration of β-glucan and protein in the EGMVs. Enhanced regeneration of keratinocyte was measured by cell viability assay and *in vitro* scratch assay. Skin regeneration-related proteins were detected by western blot analysis.

Results: Application of controlled pressure to *E. gracilis* membrane led to fabrication of the EGMVs containing β-glucans. We showed that the EGMVs were more effective to regeneration of keratinocyte and fibroblast than β-glucan only.

Conclusion: This study first introduced a technology to develop the *E. gracilis*-derived extracellular microvesicle system which is expected to pave a new way in the field of skin therapy and cosmetics.

Keywords: microalgae; extracellular vesicle; β-glucan; skin regeneration

Introduction

Cells secret extracellular vesicles (EVs) to communicate each other, deliver cargoes and modulate their physiological condition.[1] EVs are enclosed by membrane like originated cells containing various membrane proteins. Their contents such as carbohydrates, proteins and nucleotides depend on the parent cell and the microenvironment.[2] EVs are formed from the endosomal network or from the plasma membrane. EVs from the plasma membrane are referred to as microvesicles.[3] Currently, many studies are done using EVs as a natural drug delivery system due to its biocompatibility and its function as a therapeutic efficacy.

β-glucans are naturally occurred polysaccharides from fungi, algae, bacteria, cereals, and mushrooms. There are abundant forms of β-glucans different from structure, size, branching frequency and conformation.[4] For instance, β-1,3-glucans are found in bacteria and algae whereas β-1,6-glucans are found in mushroom. Due to the numerous structures, β-glucans exhibit various functions; reducing cholesterol, preventing cancer, anti- and pro-inflammation, and skin regenerative effect.[5] Especially, *E. gracilis*-derived β-1,3-glucans, form of paramylon, have shown promising results in skin regeneration. Paramylon is produced by euglenoid as a form of membrane-bound granules in the cytosol. Carbon source during cultivation affects to the content of β-glucans. Heterotrophically cultivated *E. gracilis* can accumulate 80% (w/w) paramylon of the dry weight predominantly consisting of β-1,3-glucans.[6]

Here, we proposed a method to fabricate *E. gracilis*-derived microvesicles (EGMVs) as a β glucan carrier. EGMVs can be used as a delivery system to increase the performance of β-glucan. The skin regenerative effect of the EGMVs were evaluated by cell migration and viability assay. We expect that our EGMVs could be a useful skin regenerative carrier.

Materials and Methods

Fabrication of EGMV

E. gracilis was centrifuged at 1500 g for 5 min. Then, *E. gracilis* was resuspended in PBS. The pressure about 3 psi was applied to *E. gracilis* to fabricate the EGMVs. *E. gracilis* and the EGMVs were observed by an optical microscope. The sizes of the EGMVs were measured by dynamic light scattering.

Quantitative analysis of proteins and β-glucans in EGMVs

Total concentration of protein was measured by BCA assay. BCA assay was subsequently performed according to manufacturer's instructions. The amount of β-glucan was determined by aniline blue assay. 6.7×10^{-3} M aniline blue diammonium salt, 1 M glycine, 1.25 M NaOH and 1.5 mL of 2N HCl were mixed to make working solution and stored in the dark until the mixture decolorized to yellow. Then, the EGMVs were denatured with 0.9 M NaOH at 70 ℃ for 30 min. Finally, the denatured mixture was loaded at 96-well plate with working solution at 50 ℃ for 30 min. The fluorescence intensity of solution was measured at 430/535 (excitation/emission) nm using a microplate reader.[7]

Assessment of cell viability

HaCaT cells were incubated with DMEM containing 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% gentamycin at 37 ℃. Cell viability was measured by CELLOMAX™ Viability Assay according to manufacturer's instructions. Different concentrations of the β-glucans and the EGMVs were treated to the cells for 24 h. Then, the absorbance was measured at 450 nm by microplate reader.

***In vitro* scratch wound closure assay**

HaCaT cells were seeded at the density of 1×10^5 cells per well in 12-well plate until the cells reached 100% confluence. Thereafter using sterile 200 μL pipette tips, cell monolayers were scratched. Then, the β-glucan and the EGMV were treated with 0.5% FBS in DMEM for 48 h. The gap areas were recorded by an optical microscope every 24 h.

Western blot analysis

HaCaT cells were seeded at the density of 1×10^6 cells per well in 6-well plate. After 24 h, the β-glucan and the EGMV were treated with 0.5% FBS in DMEM for 24 h. Then, the cells were washed with PBS and 1x RIPA and 1x protease inhibitor were treated for cell lysis. The cell lysates were loaded to SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes. Then, the membranes were blocked with 5% non-fat milk in Tris-buffered saline and incubated overnight at 4 ℃ with primary antibodies; cytokeratin 10 (ab111447), Src (ab47405), AQP3 (ab125219) from Abcam (UK) and cytokeratin 14 (PA5-16722) from Invitrogen. The immunoreactive proteins were detected by using chemiluminescent reagent, EzWestLumi Plus. Then the proteins were quantified by CS Analyzer 4.

Results

The presence of a mechanically strong cell wall commonly disturbs the efficient product of EVs. However, *E. gracilis* has been known that it has the GRAS (Generally recognized as safe) status while not having a cell wall, which inspired us to select it as a starting cell material. The strategy for fabrication of the EGMVs having β-1, 3-glucan is illustrated in Fig. 1. About 3 psi pressure was applied to *E. gracilis* to fabricate EGMVs. As a result, the membrane of the EGMVs is very similar to *E. gracilis so* that various biocompounds from *E. gracilis* like fibrous carbohydrates, amino acids, and mRNAs can be encapsulated in EGMVs.

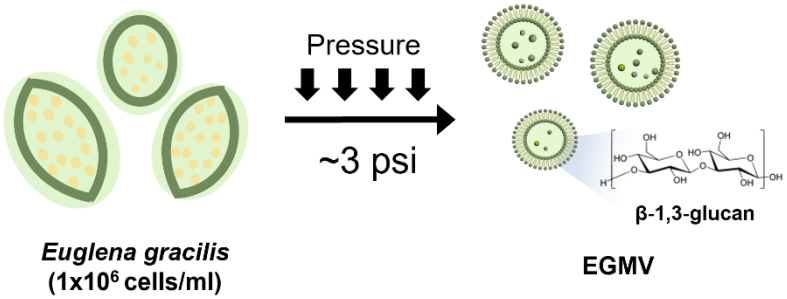

Figure 1. Schematic strategy for fabrication of EGMV.

EGMV formation was monitored using optical microscopy (Fig. 2a-b). When the pressure was applied to *E. gracilis*, there are no longer visible cells and only stable particles were found in the micrometer range. The size of EGMV was ~1 μm, which is similar to microvesicle (Fig. 2c). Because the size is decreased compared to *E. gracilis,* both concentrations of protein and β-glucan in EGMV were decreased. In EGMV, 50% of protein and 1% of β-glucan were remained compared to cells. β-glucan concentration was measured as 20 μg/ml by aniline blue assay. Therefore, control group is composed of the same amount, 20 μg/ml β-glucan. To confirm the efficacy of decreased amount of β-glucan, we treated HaCaT cells with EGMV for 24 h and observed their cell viability by MTT assay varying the β-glucan concentration (Fig. 2e). As the amount of β-glucan increased, the cell viability increased. Compared to the group treated only β-glucan, the group treated EGMV showed ~15 % increased viability.

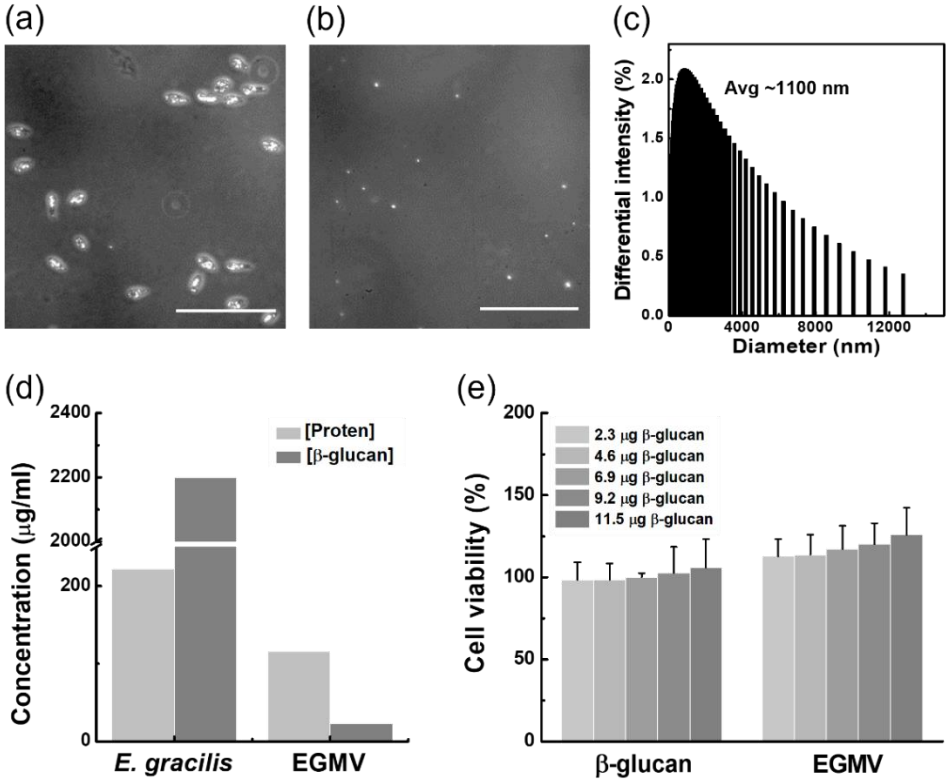

Figure 2. Optical microscope images of (a) *E. gracilis* and (b) EGMV. The scale bars are 20 μm. (c) Particle size distribution of EGMV. (d) Protein and β-glucan concentrations of *E. gracilis* and EGMV. (e) Cell viability of different concentrations of β-glucan and EGMV in HaCaT cells.

Cell migration ability of β-glucan was evaluated by an *in vitro* scratch assay for HaCaT cells (Fig. 3a-b). After HaCaT cells were incubated for 24 h, the area of EGMV treatment decreased more than the area of free β-glucan treatment. After 48 h, the difference between them became significantly pronounced. The difference between two groups was about 27 % at 48 h treatment. In this process, β-glucan activated growth factors to promote cell migration and synthesize collagen.[8] Continuously, we conducted a western blot analysis to confirm the expression of skin regenerative proteins; Src, cytokeratin 10 (K10), cytokeratin 14 (K14) and aquaporin 3 (AQP3). All protein expressions in HaCaT cells were more increased about 20% after treatment with EGMV than treatment with free β-glucan.

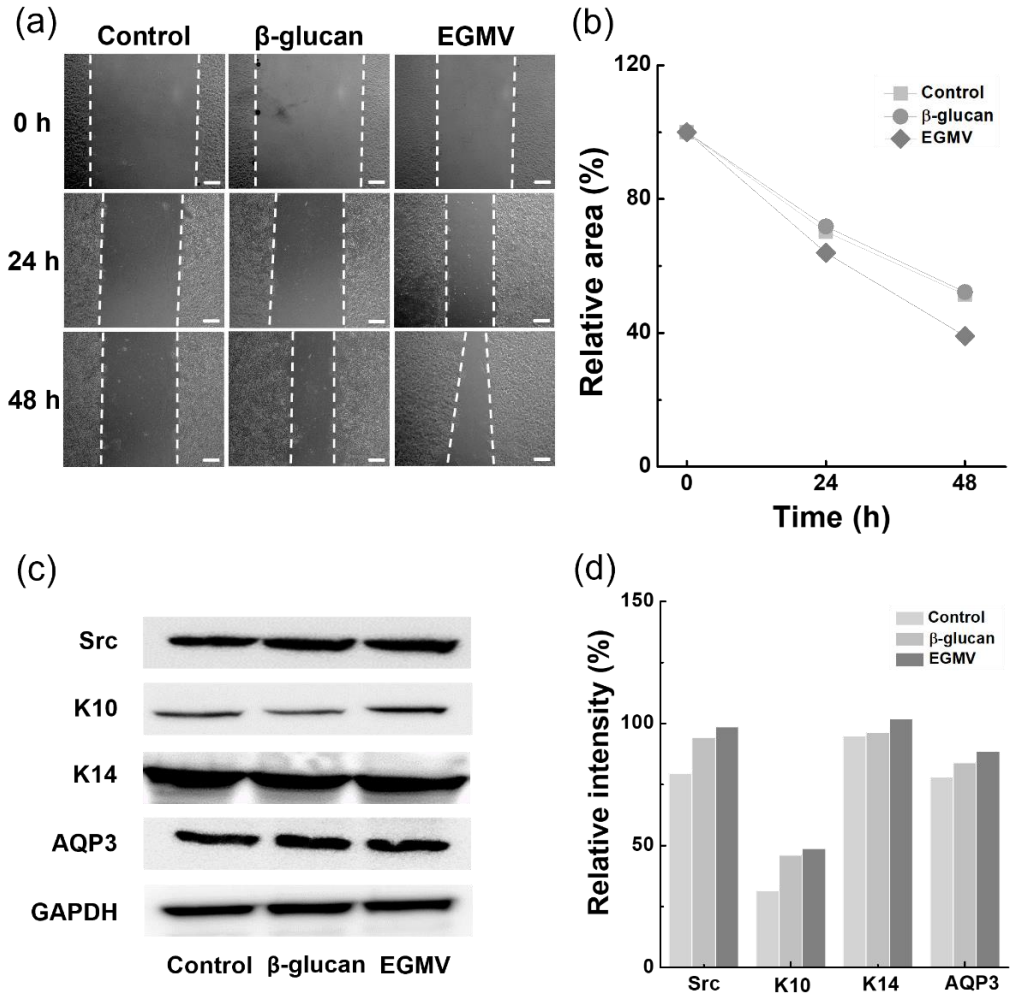

Figure 3. (a) Representative images of scratch assay treating β-glucan and EGMV as a function of time. The scale bars are 50 μm. (b) Calculated wound closure area of bright-field microscopy images. (c) Detection of skin regeneration-related protein treating β-glucan and EGMV by western blot. (d) Densitometric analysis of each protein bands.

β-glucan activates Src signaling pathway to induce keratinocyte proliferation and migration by synthesizing cytokeratin like K10 and K14.[9] For fibroblast, β-glucan accelerates the collagen synthesis. Because EGMV contains lots of bioactive materials that activates various biological reactions more research is needed to prove the protein related to β-glucan mechanism and measurement of collagen.

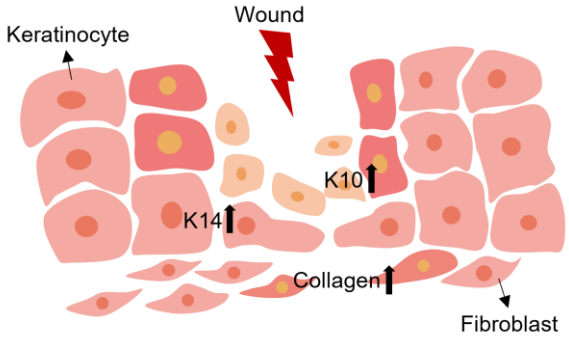

Figure 4. Schematic illustration for the effect of β-glucan on keratinocyte and fibroblast.

Discussion

We firstly propose *E. gracilis*-derived microvesicles as a β-glucan carrier. We elucidated that the EGMVs could encapsulate given amounts of β-glucans as a cargo which is effective for skin regeneration. As expected, the EGMVs were more effective than free β-glucan on keratinocyte proliferation and migration. The EGMVs could be a protection barrier against glucanase and an effective for drug delivery.

Conclusion

In summary, we used a pressurization technology to *E. gracilis* for fabrication of skin-regenerative microvesicles. We showed that although *E. gracilis* contains β-glucan as the form of granule, paramylon, the paramylon is separated to form fiber cleavages that can be easily and efficiently taken up by the cells. The *E. gracilis*-derived β-glucan induces the production of growth factors which are essential for promoting collagen biosynthesis in the skin. Taking advantage of this, we successfully demonstrated the EGMVs are truly useful for enhancing skin regeneration, thus allowing them to have great potential in the field of skin therapy and cosmetics.

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Conflict of Interest Statement

None.

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