学 位 論 文 の 要 旨

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学 位 論 文 名　Bullous Pemphigoid IgG Induces Cell Dysfunction and Enhances the Motility of Epidermal Keratinocytes via Rac1/Proteasome Activation

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論 文 内 容 の 要 旨

INTRODUCTION

Bullous pemphigoid (BP) is a skin-specific autoimmune disease characterized by subepidermal blisters. The autoimmune mechanisms involved in BP have been well discussed based on clinical and experiential evidence. IgG is the principal antibody involved, which recognizes the non-collagenous 16a domain (NC16a) of type XVII collagen (ColXVII). Binding of the specific IgG to ColXVII-NC16a is the most essential initial event in BP, and it is considered to contribute to intra-lamina lucida blistering in subjects with BP.

Recently, the binding of BP IgG to ColXVII has been reported to cause internalization of the immune complex by forming macropinosomes. Thus far, the changes in the morphology of keratinocytes during BP IgG-induced macropinosome formation are incompletely understood, and knowledge regarding macropinosome formation in the pathogenesis of BP is still lacking. The aim of this study was to clarify the morphological and functional changes in human keratinocytes incubated with BP IgG and the subsequent effects of inhibitors of macropinosome formation on these morphological and functional events.

MATERIALS AND METHODS

Normal human epidermal keratinocytes (NHEKs) were cultured in serum-free media supplemented with growth factors, 1% penicillin-streptomycin, and 25 ng/ml amphotericin B at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were used after two to six passages in all experiments. Plasma samples were obtained from 3 patients with BP, and eluents were obtained by a double filtration plasmapheresis treatment. BP IgGs fractions were isolated from plasma using a HiTrap Protein G HP column with a fast protein liquid chromatography system. The functions of BP IgGs were confirmed using indirect immunofluorescence staining of the

salt-spilt human skin and a ColXVII-NC16a chemiluminescent enzyme immunoassay test. Immunoblotting using the normal human epidermal extracts confirmed that the BP IgGs used in this study only reacted with ColXVII (180 kDa) and not with other antigens. The pooled IgGs from healthy people (normal IgG) were used as control IgGs.

NHEKs were co-cultured with BP IgGs or normal IgG in the presence or absence of following inhibitors: cytochalasin D (inhibitor of actin-cofilin interaction), NSC23766 (inhibitor of Rac1 activity) and MG132 (proteasome inhibitor). Cell morphologic and function changes were analyzed by flow cytometry assay, immunostaining assay, time-lapse microscope observation, cell counting assay, transmission electron microscope (TEM) and scanning electron microscope (SEM) observation, LysoTracker® Green DND-26 staining assay, MitoTracker® Red CMXRos staining assay, cellular reactive oxygen species (ROS) assay, mitochondrial membrane potential assay, C_{12}-resazurin/SYTOX® green staining assay, and cell motility assay. ANOVA or Student's t-test was used to compare the means, and differences were deemed significant when the calculated p-value was <0.05. All statistical analyses were performed with the R language. The study protocol was approved by the Research Ethics Committee of Shimane University.

RESULTS AND DISCUSSION

In the flow cytometry analysis, the cell surface IgG binding and ColXVII expression on the surface of BP IgGs-stimulated NHEKs decreased over time, whereas normal IgG-stimulated cells was not obviously altered. Based on the immunostaining assay, the BP IgGs-staining was observed to be internalized into NHEKs' cytoplasmic region at 2 h. Phase-contrast images indicated BP IgGs-induced formation of typical macropinosomes, and the BP IgG-stimulated cells exhibited a significant increase in the number of cells containing macropinosomes compared to normal IgG- or untreated cells.

The cell counting assay with living cells showed that BP IgGs significantly decreased the number of attached cells (reduced by approximately 40%) compared to the cells treated with normal IgG or untreated cells, and the effects were evident 2 h after the incubation. The sizes of the NHEKs increased after the cells were incubated with BP IgGs for 2 h. The SEM examination revealed that the BP IgGs-stimulated cells exhibited alterations in the cell membrane structure. The TEM examination revealed larger vesicles with villi-like structures inside the cytoplasmic space in BP IgGs-treated NHEKs, and that the BP IgGs-stimulated cells accumulated lysosomes and/or autophagosomes, whereas cell nuclei of these cells showed neither damage nor chromatin condensation. Moreover, BP IgGs-stimulated cells contained a greater number intracellular LysoTracker® Green DND-26-labeled dots with high fluorescent intensity.

MitoTracker® Red CMXRos staining assay showed that the staining intensity of mitochondria in BP IgGs-treated cells decreased after 16 h BP IgGs-incubation, although the

number of stained mitochondria per cell did not differ between BP IgGs-treated cells and normal IgG-treated or untreated cells. ROS were generated 6 h after BP IgGs were added to the culture, whereas normal IgG did not increase ROS levels. By performing JC-1 staining to analyze the mitochondrial membrane potential ($\Delta\Psi m$), dysfunctional mitochondria with a dissipation of $\Delta\Psi m$, as evidenced by the increased number of JC-1 monomers, were pronounced in BP IgGs-treated NHEKs at 20 h. The NHEKs that had been incubated with BP IgGs for 16 h were used to perform SYTOX® green/C_{12}-resazurin staining assay. A greater percentage of BP IgG-stimulated cells was stained with SYTOX® green and a relatively lower percentage was stained with C_{12}- resazurin than normal IgG-stimulated or untreated cells.

In cell motility assay, throughout the 6 h observation of live cells, BP IgGs-treated cells showed increased cell motility compared with normal IgG-stimulated and untreated cells. Notably, both the migration distance and velocity increased.

Application of cytochalasin D inhibited the internalization of the BP IgG-ColXVII immune complex in BP IgGs-stimulated NHEKs, but did neither protect the impaired adhesion, the compromised cell plasma membranes, nor the increased motility of the NHEKs treated with BP IgGs. Application of NSC23766 prevented the BP IgGs-ColXVII immune complex internalization and the compromised cell plasma membranes of the BP IgGs-stimulated NHEKs, suggesting that Rac 1 activation is involved in the BP IgGs-stimulated keratinocyte dysfunction. Surprisingly, NSC23766 did neither protect the impaired adhesion nor the increased motility of the NHEKs treated with BP IgGs. Application of MG132 did not prevent BP IgGs-induced internalization of the BP IgG-ColXVII immune complex in BP IgGs-stimulated NHEKs. However, MG132 application protected NHEKs from the BP IgGs-induced cell dysfunction. Additionally, MG132 application slightly decreased the migration of BP IgGs-treated cells, suggesting an involvement of proteasome activation in the NHEKs treated with BP IgGs.

Those findings support the hypothesis that BP IgGs directly cause subepidermal bulla formation in patients with BP through the internalization of the BP IgG-ColXVII immune complex followed by impaired adhesion, increased motility, and cell dysfunction of the keratinocytes.

CONCLUSION

We conclude that the persistent stimulation with BP IgG-induced macropinosome formation in keratinocyte results in a fragile plasma membrane, intercellular vesicle formation, gathering of lysosomes, and promote the accumulation of ROS, which finally contribute to mitochondrial dysfunction. The BP IgG-induced keratinocyte alteration triggers the immune system to digest the basal keratinocytes, which causes skin blister formation along with basement membrane zone.