論文の内容の要旨

論文題目　Acylhomoserine lactone improves the impaired basement membrane formation in the epithelializing tissue during cutaneous wound healing in hyperglycemic rats
（高血糖ラット皮膚欠損創の治癒過程における上皮基底膜の形成不全に及ぼす Acylhomoserine Lactone の改善効果）

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The recent changes in the social environment and lifestyle have resulted in a dramatic increase in the incidence of diabetes mellitus (DM) in both the developing and developed nations. The number of people suffering from DM worldwide is predicted to increase from 177 million in 2000 to 366 million by 2030, and it has been reported that approximately 10% of patients with type 2 diabetes have some type of complications such as retinopathy, angiopathy and neuropathy. These data indicate that DM is a major threat to public health throughout the world.

One of the most important complications in patients with DM is impaired wound healing. The prolonged period of wound healing increases the risk for wound infection, medical costs and decreases the quality of life for diabetic patients. Nurses are frequently confronted with chronic wounds in which no advancement and tylosis of the wound edges are observed in diabetic patients, despite the application of advanced treatments for chronic wounds. These clinical experiences suggest that there is inhibition of re-epithelialization in diabetic patients. However, the studies on the mechanisms of the delayed re-epithelialization under diabetic conditions is limited.

The advancement of re-epithelialization is mainly influenced by the proliferation and migration of epidermal keratinocytes. The basement membrane components such as laminin 5 (LM5), fibronectin (FN) and type IV collagen (Col4) are important scaffolds for these cellular functions. Recently, it has been identified that extracellular matrices (ECMs) also play the role of functional molecules to regulate the cellular proliferation and migration directly. Moreover, a few reports revealed the abnormal structure of basement membrane in wounds of diabetic patients. However, so far, the relationship between the structural abnormalities of the basement membrane and keratinocyte functions has not been clarified in diabetes. In the present study, I hypothesized that the impairment of basement membrane induced the delayed re-epithelialization under the diabetic condition.

Many researchers have recognized that it is difficult to investigate wound healing in diabetic patients because of the wide range of clinical factors in such patients. In order to clarify the mechanisms underlying the delayed re-epithelialization in diabetic patients and to consider the development of a novel therapeutic approach, I focused on the sole effects of hyperglycemia on re-epithelialization, since the continuously elevated blood glucose level plays a central role in the pathogenesis of various complications of DM, including delayed wound healing. The effects of hyperglycemia itself on the structure of basement membrane also have not been elucidated.

This work was primarily conducted to uncover the relationship between the abnormalities of the basement membrane and the delayed re-epithelialization of cutaneous wounds in hyperglycemic model rats. Furthermore, I attempt to develop a novel advanced wound management technology to improve the abnormalities of the basement membrane and to promote the re-epithelialization under hyperglycemic conditions.

In the chapter 1, in order to elucidate the relationship between the impairment of the basement membrane and the cellular functions of keratinocytes under hyperglycemic condition, streptozotocin-induced acute hyperglycemic model rat was used. A full-thickness round wound (diameter 2 cm) was created on their flank region. Then macroscopic appearance of the wounds, the wound area, and the complete wound closure day were assessed. The tissue of wound and surrounding area was harvested on post-wounding day (PWD) 7. Then the histological (HE staining) as well as immunohistochemical analyses (the structure of basement membrane: LM5, FN, Col4; the proliferation of keratinocytes: Ki67) were performed. The reverse transcription-polymerase chain reaction (RT-PCR) for the components proteins of the basement membrane (LM5, FN, Col4) and their degrading enzyme (MMPs, TIMPs) was also conducted.

Compared with the control group, the hyperglycemic group showed significantly delayed wound closure day, larger wound area from PWD 7 to 9, and shorter length of the re-epithelializing tissue on PWD 7. Histological abnormalities of the regenerating epidermis such as the invagination into the granulation tissue and tylosis were observed in all of the hyperglycemic rats, and it was not observed in any of the control rats. The immunohistochemical analysis for Ki67 expression indicated that there was increased proliferation of keratinocytes in the limited invaginating area, while it was rare in the flat area in the hyperglycemic rats. On the other hand, the proliferating cells were uniformly distributed in the basal layer of the regenerating epidermis in control rats. The results of RT-PCR revealed that the synthesis of LM5 was inhibited, and that the expression levels of MMPs which target the three major components of the basement membrane were elevated in the wound sites of the hyperglycemic rats. The results of the immunohistochemical analysis for ECM components of the basement membrane indicated that there were hyperglycemia-unique abnormalities of the basement membrane, including the fragmentation, and the wavy and thickened shape of the basement membrane with decreased immunoreactivity for ECMs, termed as wavy structure. In particular, fragmentation of the basement membrane was more frequently observed around the invagination of the regenerating epidermis. These results strongly suggest that the delayed re-epithelialization and histological abnormalities were mainly due to the defects of the basement membrane, and the improvement of the impaired basement membrane functions may represent a possible therapeutic strategy to enhance re-epithelialization in hyperglycemia.

In the chapter 2, I focused on acylhomoserine lactone (AHL) as a candidate reagent to promote re-epithelialization under hyperglycemia. Acylhomoserine lactone is the quorum sensing (QS)-related signal molecule, called as an autoinducer, in *Pseudomonas aeruginosa*. Recently, some researchers have focused on the regulation of gene expression in mammalian host cells by bacterial AHL, which they have named “inter-kingdom signaling”. The previous study of our laboratory indicated that AHL accelerates cutaneous wound healing through myofibroblast differentiation in rats. Another research revealed that the inoculation of animals with *P. aeruginosa* accelerated the re-epithelialization of cutaneous wounds. These findings suggested that AHL signaling may underlie to enhanced re-epithelialization induced by inoculation with *P. aeruginosa.*

However, the AHL signaling pathway in mammalian cells has not been fully elucidated. The activation of the MAPK (mitogen-activated protein kinase)/NF-κB (nuclear factor-kappa B) pathway and binding with peroxisome proliferator activated receptor gamma (PPARγ) have been identified as the possible mechanisms responsible for the AHL-mediated regulation of gene expression in mammalian cells. It was also reported that the expression of PPARγ was abundantly detected in keratinocytes, involving in normalizing epidermal homeostasis in human and murine models. Another previous study histologically demonstrated that there was increased LM expression in the pancreas in mice with chronic pancreatitis following treatment with a PPARγ agonist, troglitazone. The integration of these knowledge prompted me to hypothesize that topical AHL administration may represent a possible treatment to improve the impaired basement membrane under hyperglycemic conditions.

In order to reveal the beneficial effects of AHL on the impaired re-epithelialization resulting from the improvement of structural abnormalities of the basement membrane, I performed the following experiments. First, fetal rat skin keratinocyte (FRSK) cell line was cultured in three types of treatment media: the normal growth medium (no treatment group), the growth medium supplemented with 0.1% DMSO (solvent group) or 10 μM AHL (AHL group). The gene expressions of the components of the basement membrane and their degrading enzymes were analyzed *in vitro*. Furthermore, the effects of AHL treatment on impaired re-epithelialization in hyperglycemic rats were analyzed. Two full-thickness wounds were created on the both sides of each hyperglycemic rat. The right side wounds were treated with AHL whereas the left sides were with solvent. The delayed re-epithelialization was evaluated with the macroscopic observation, and the effect of AHL on the abnormalities of regenerating epidermis was analyzed by histological and immunohistochemical methods as in chapter 1.

In vitro, significant increase of the mRNA expression of *Lm5* was observed in the AHL-treatment group. *In vivo*, significant enhancement of wound closure in the AHL-treated group was observed from post-wounding day 8, and the period for complete wound closure was significantly shortened in the AHL-treated wounds compared with the control wounds. The findings of histological and immunohistochemical analyses on the PWD 7 showed that the invagination of the regenerating epidermis, fragmentation and wavy structure of the basement membrane, which were the hyperglycemia-related abnormalities identified in chapter 1, were significantly repaired in the AHL-treated wounds, while these abnormalities were frequently observed in the control wounds. Moreover, the proliferation analysis of keratinocytes indicated the uniform distribution of Ki67-positive keratinocytes in the basal layer of the regenerating epidermis in the AHL group. The improvement of these abnormalities by AHL treatment supported my hypothesis that the impairment of basement membrane induced the delayed re-epithelialization, and suggested that AHL administration normalized, rather than enhanced, the re-epithelialization under the hyperglycemic condition.

This work demonstrated a new mechanism of the delayed re-epithelialization of the chronic wounds in hyperglycemia, possibly due to the structural abnormalities, namely fragmentation and a wavy structure of the basement membrane. In the searching for the improvement methods for the abnormalities in re-epithelialization under hyperglycemia, I identified a new chemical compound (AHL) which could improve the abnormalities of basement membrane, including enhancement of LM5 expression, and accelerate the re-epithelialization under

hyperglycemic conditions. These findings suggest that AHL administration is a possible therapeutic approach for the treatment of ulcers under conditions of hyperglycemia.