Development of biomarkers for the Wound Fluid RT-PCR method to detect critically colonized and infected wounds

論文の内容の要旨

論文題目　Development of biomarkers for the Wound Fluid RT-PCR method to detect critically colonized and infected wounds
（滲出液 RT-PCR 法によるクリティカルコロナイゼーション及び感染創傷検出のためのバイオマーカーの開発）

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INTRODUCTION

Wound infection is a form of host damage resulting from an imbalance between pathogen virulence and the host immune response. The wound infection continuum is a concept that consists of four sequential conditions exist in open wounds resulting from the level of bacterial bioburden present. According to this concept, wound contamination is the presence of non-replicating microorganisms in the wound. Wound colonization is the presence of replicating microorganisms adherent to the wound that are not causing injury to the host. Critical colonization causes delayed healing without inducing an overt inflammatory response. Wound infection is the presence of replicating microorganisms within a wound with subsequent host injury accompanied by inflammation. Although it is important to detect the clinically abnormal states of critical colonization and infection that require immediate intervention, no objective indicators are yet available to do so.

In recent years, critical colonization has emerged as a problematic status in clinical situations as there are no typical clinical signs and symptoms. Moreover the representative manifestation of critically colonized wounds is delayed healing, so that it is almost impossible to diagnose this early. Therefore, it is imperative to develop a rapid, accurate method to detect delayed healing caused by the presence of bacteria. Such indicators should be non-invasive, objective, prompt, and capable of detecting both critical colonization and infection.

We previously established a novel methodology in rats, the wound fluid reverse-transcription polymerase chain reaction (RT-PCR) technique, which uses RT-PCR to search for biomarkers of infection in cells contained within wound fluids. We successfully identified marker genes that were specifically expressed in colonized or infected wounds, and combined them for use as a precise rat biomarker of infection. However, these marker genes were identified from a monomicrobial infection rat model and were not proven to work in clinical samples to detect such problematic bacteria-related wound states.

The purpose of this study, therefore, was to apply this novel approach to clinical settings; in particular to establish biomarkers for bacteria-related delayed healing in humans. We aimed to identify marker genes that show higher expression in critically colonized and infected wounds in clinical wound fluid samples to differentiate between colonization (normal healing) and critical colonization (delayed healing) or worse using the wound fluid RT-PCR method. The strategy we pursued to achieve this goal included: 1) the development of animal models according to the infection continuum; 2) whole rat genome microarray expression profiling of the cells in wound fluids obtained from these animal models to generate lists of candidate marker genes; 3) the testing of the candidate marker gene expression levels in clinical wound fluid samples to determine whether they can be used as markers for detecting critically colonized and infected wounds in humans.

CHAPTER 1. *Establishing a wound animal model and its validation*

BACKGROUND

To identify biomarkers for distinguishing critically colonized and infected wounds from those with other statuses, we first needed to establish animal models according to the infection continuum. Since we have previously developed animal models of wound colonization and infection, the primary purpose of this chapter was to establish and validate an animal model of critical colonization. We hypothesized that the same experimental strategy used previously for our colonization and infection models would be applicable for the creation of a critical colonization model because this is conceptually located at the intermediate stage between colonization and infection. We therefore administered a bacterial concentration halfway between the concentrations used for generating colonization and infection models. We then validated the critical colonization model according to the definitions of the wound infection continuum.

MATERIALS AND METHODS

We created full-thickness wounds on the flank region of male Sprague-Dawley rats (approximately 6 months old) and inoculated different concentrations of *Pseudomonas aeruginosa* to produce the following four groups: control (optical density at 600 nm (OD_{600}) = 0.0; phosphate buffered saline), colonization (OD_{600} = 0.5; 2.06×10^9 CFU/mL), critical colonization (OD_{600} = 0.75; 3.24×10^9 CFU/mL), and infection (OD_{600} = 1.0; 4.12×10^9 CFU/mL). Histological characterization was investigated by preparing paraffin-embedded tissue sections for scanning electron microscopy (SEM). Swelling of the periwound area was determined in hematoxylin and eosin-stained sections. The areas of the wound and redness in the periwound skin were evaluated by digital image analyses.

RESULTS

Gross observation revealed that the wound contraction and the area reduction were limited in the critical colonization group and that the wounds were larger and deeper compared with the control group on day 6 after wounding. The colonization group showed similar macroscopic characteristics to the control group. There was no obvious redness or swelling in the surrounding skin on day 6 in either the critical colonization group or the colonization group. In the infection group, obvious signs of infection such as persisting redness in the periwound skin, a spreading necrotic area, increased wound depth, pocketing, and a purulent exudate were observed. Wound area reduction revealed delayed healing in the critical colonization group compared with the control group. SEM observation identified the presence of a bacterial biofilm in both the critical colonization and infection groups. Although rod-shaped bacterial structures were observed in the critical colonization group, these were encased in extracellular polymeric substances. Many bacterial cells resided in a matrix in the infection group, however, the bacterial cells were protruding from the matrixes and exposed.

DISCUSSION

We successfully developed a novel rat model of critical colonization presenting delayed healing with no obvious signs of inflammation. It showed superficial bacterial growth as previously mentioned in terms of critical colonization. Investigation of the model also revealed that there was aggregated inflammatory cells in the subcutaneous tissues. SEM observation identified the distinct biofilm phenotypes in the critical colonization and infection groups, suggesting that the persistent inflammation under the skin accompanying biofilms with bacteria encased in extracellular polymeric substances was the pathogenesis of critical colonization.

CHAPTER 2. *Identification of candidate marker genes involved in rat cells from Pseudomonas aeruginosa-infected wound fluid*

BACKGROUND

Little is known about the cell populations in wound fluids, especially, in those wounds with different bacteria-related states. Therefore, this basic information should be elucidated before performing a DNA microarray of the collective cells contained in wound fluids. The purpose of this study was: (1) to investigate the cell population in wound fluids of each animal models (control, colonization, critical colonization, and infection) to examine the relationship with bacteria-related wound conditions; (2) to perform a DNA microarray analysis of the gene expression profile after host cell sorting; and (3) to generate lists of candidate marker genes to determine if these could be used as clinical markers for detecting critically colonized and infected wounds.

MATERIALS AND METHODS

The wounds were covered with transparent film dressings and the pooled wound fluid was collected using syringes with needles. The granulocytes, helper T cells, cytotoxic T cells, regulatory T cells, and B cells were identified using relevant rat antibodies (fluorescein isothiocyanate-conjugated anti-rat granulocytes antibody, phycoerythrin-cyanin 7-conjugated anti-rat CD4 antibody, chlorophyll protein-conjugated anti-rat CD8a antibody, phycoerythrin-conjugated anti-rat CD25 antibody, and allophycocyanin-conjugated anti-rat CD45RA antibody, respectively) and counted with a flow cytometer. Cells from wound fluids extracted on day 6 were then sorted by flow cytometry and used for total RNA extraction. Whole rat genome microarray expression profiling was employed for the above-mentioned four groups to identify the gene expression profile with and without bacteria-related delayed healing. The microarray data were analyzed by GeneSpring software to obtain lists of candidate marker genes.

RESULTS

Granulocyte counts in the wound fluids from the infection group were the highest of all four groups after day 4. Surprisingly, there were no T cells in the infection group wound fluid after the day 4, although small numbers of T cells were present in the critical colonization group.

A total of 300 candidate marker genes were identified that expressed a less than 1.0-fold change in the colonization group compared with the control, a more than 2.0-fold change in the critical colonization group, and a more than 10-fold change in the infection group (List 1). List 2 consisted of 250 candidate marker genes, and was generated by the following conditions: a 1.0–1.5-fold change in the colonization group, a ≥2.0-fold change in the critical colonization group, and a ≥10.0-fold change in the infection group. These were ranked by the infection fold-change in descending order.

DISCUSSION

The revelation that there were no lymphocytes in wound fluids from the infection model, but some in the critical colonization model indicates that cells are not sufficient as a biomarker because we require and indicator more specific to the critical colonization and infection. We decided to proceed with DNA microarray analysis of total cell population rather than only part of populations because the differences in cell population were existed but limited in small populations. We obtained gene expression profile datasets of wound fluid samples from four distinct bacteria-related wound statuses from the results of the DNA microarray. These were used to successfully generated lists of the candidate marker genes.

CHAPTER 3. *Identification of marker genes for critically colonized and infected wounds in humans*

BACKGROUND

The final step was to measure the expression levels of candidate marker genes identified in Chapter 2 to determine whether they are suitable as markers of critically colonized and infected wounds for clinical samples of wound fluid in humans.

MATERIALS AND METHODS

Eleven patients with chronic wounds were entered into this study. Wounds were judged retrospectively and divided into the normal healing, critical colonization, and infection based on the wound area, quantitative bacterial counts, and clinical evaluations. We collected used gauzes from these patients containing wound fluid that had been secreted over an approximately 24-h period. The gauze samples were immersed into 1 × phosphate-buffered saline and cells were collected from the retrieved dressings and total RNA was extracted. Then the expression of candidate marker genes were analyzed from the collected gauze in each group. Real-time RT-PCR was used to investigate the gene expression levels of the top 20 genes from the candidate marker gene lists in human wound fluid samples.

RESULTS

Real-time RT-PCR was performed to quantitatively estimate the mRNA expression of target genes in humans. Of the 20 tested candidate marker genes, five genes were not expressed in the normal healing group (0/4 samples), but showed higher expression levels in the critical colonization and infection groups (7/7 samples). In addition, expression of one gene was not detected in normal healing (0/4) but detected in critically colonized and infected wound samples (6/7). The five genes had 100% sensitivity and 100% specificity for detection of critical colonization or infection. Likewise, another gene had 85% sensitivity and 100% specificity. Moreover, the critical colonization group showed significantly higher C*t* values (i.e., lower expression levels) than those of the infection group for four genes out of above-mentioned five marker genes.

DISCUSSION

Our results revealed that analysis of the expression of a combination of genes could be used to differentiate critically colonized and infected wounds from other wound statuses. We have identified a number of marker genes that can distinguish normal healing and critical colonization in clinical settings by the presence or absence of their expression. Moreover, it appears that critical colonization and infection can also be distinguished quantitatively. This study shows the possibility of using these markers clinically even for wounds with polymicrobial infections.

CONCLUSION

We identified a combination of novel marker genes that can distinguish critically colonized and infected wounds from other wound conditions in human wound fluid samples. It is suggested that this successful identification was achieved by the proper strategy: establishing stringent animal models, performing DNA microarray experiments, and examining the efficacy of marker genes by real-time RT-PCR in target samples. More detailed analysis in a larger number of patients will enable us to confirm the efficacy of these biomarkers. We anticipate that they will be applied to future clinical settings in the form of a handy diagnostic kit.