行政院國家科學委員會專題研究計畫　成果報告

成人型牙周炎致病菌(Porphyromonas gingivalis)調控人類心臟血管內皮細胞之基因表現之基因晶片研究

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-038-011-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
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計畫主持人：周幸華

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行政院國家科學委員會專題研究計畫成果報告

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主持人：周幸華　台北醫學大學牙醫學系
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一、中文摘要

成人型牙周炎為牙齦組織之慢性發炎反應, 其病因主要為牙齦組織受到特定的牙周致病菌之感染，因而造成牙齦組織之破壞 (1)。在成人型牙周炎病患的牙周囊袋或發炎的牙齦組織內，經常可檢測出 *Porphyromonas gingivalis* (*P. gingivalis*)。另外，靈長類動物之牙齦組織，接種 *P. gingivalis* 之後也會造成動物之牙周炎。因此，*P. gingivalis* 被認為是成人型牙周炎的主要致病菌之一 (3)。

P. gingivalis 造成牙周炎的致病因子很多，其中其細菌表面之纖毛(fimbriae) 為 *P. gingivalis* 附著於宿主細胞及入侵宿主細胞之最重要的菌體結構之一 (17-22)。這些纖毛不僅能幫助 *P. gingivalis* 入侵宿主的牙齦表皮細胞及牙齦組織(15)，甚至能活化宿主的多項免疫防禦系統。例如，調控宿主的牙齦表皮細胞或牙齦纖維母細胞或宿主的防禦免疫細胞分泌炎性或趨化性介質來控制宿主之免疫發炎反應(20)。另外，本計劃申請人等的研究也指出 *P. gingivalis* 能侵入人類之血管內皮細胞並調控人類血管內皮細胞表現細胞接著分子(cell adhesion molecules)及分泌趨化性介質(如 IL-8)(26, 27)。但是，纖毛基因剔除之 *P. gingivalis* 變異株(*fimA* gene insertional mutant)則無刺激人類血管內皮細胞表現細胞接著分子(cell adhesion molecules)及分泌趨化性介質之作用。至目前為止，探討 *P. gingivalis* 調控宿主細胞基因表現之研究多侷限於少量特定之基因研究。隨著人體基因資料庫之擴張及基因晶片技術之發展已趨成熟及普及。本研究計劃利用 Affymetrix 公司生產的 HG-U95 基因晶片來分析並建立 *P. gingivalis* 及其纖毛基因剔除之變異株感染人類血管內皮細胞之後，血管內皮細胞基因表現之資料庫。本研究計劃共分為三部分:第一是利用基因晶片來建立 wild-type *P. gingivalis* 感染人類血管內皮細胞之後，血管內皮細胞基因表現之資料庫；第二是利用纖毛基因剔除之 *P. gingivalis* 變異株來建立其感染人類血管內皮細胞之後，血管內皮細胞基因表現之資料庫。藉由兩資料庫之比較將可達到本計劃之第三個目的: 建立 *P. gingivalis* fimbriae 調控血管內皮細胞基因表現之資料庫。

Keywords: Porphyromonas gingivalis, fimbriae, gene chips, RNase Protection Assay

二、英文摘要

Adult periodontitis is a chronic inflammatory disease that is caused by a mix infection of bacteria (1). The severity of the disease is based both on the specific organisms isolated from infected sites, as well as the immune status of the host. Porphyromonas gingivalis (*P. gingivalis*) which has been implicated as a major etiological agent in the development of adult periodontitis is a highly invasive pathogen. *P. gingivalis* adhere to host cells by its surface structures such as fimbriae. Several studies have shown the role of *P. gingivalis* fimbriae in mediating adherence to host cells (17-19); *P. gingivalis fimA* insertional mutant (DPG3) has also been shown to loss its adhesiveness and invasiveness to oral epithelial cells or endothelial cells (10,15). In addition, We have recently shown that fimbriae stimulate the expression of inflammatory cytokine expression in endothelial cells (21). These studies strongly suggested that fimbriae play a crucial role in *P. gingivalis* interaction with host cells.

Periodontal diseases have recently been associated with cardiovascular disease and preterm delivery of low birth weight infants (10, 11-13). Transcriptional activity of endothelial

cells following infection with *P. gingivalis* has been reported. Induction of various molecules with immunological activity including cell adhesion molecules and inflammatory cytokines such as IL-8 and MCP-1, have been observed (21,22). These findings are consistent with a role of *P. gingivalis* in the pathogenesis of atherosclerosis. These reports, however, focus only on a small number of genes encoding immunoregulatory proteins that may represent a small subset of inducible genes that are activated in endothelial cells following infection with *P. gingivalis.*

Microarray technology is now readily available and allows characterization of the mRNA levels for a large number of genes simultaneously, thus providing a useful tool to identify broad spectrum changes in gene expression in cells in response to a given stimulus (23-25). The present study use oligonucleotide microarrays to analyze mRNA expression for a large number of genes in human aortic endothelial cells (HAEC) following infection with *P. gingivalis.* In addition, we will utilize *P. gingivalis fimA* mutant to study the effects of *P. gingivalis* fimbriae or *P. gingivalis* invasion on the gene expression status of infected HAEC.

Keywords: Porphyromonas gingivalis, fimbriae, microarray, RNase Protection Assay.

三、計畫緣由與目的

Adult periodontitis is a bacterially induced chronic inflammatory disease that leads to inflammation of the gingiva, destruction of the periodontal tissues and, in severe cases, loss of alveolar bone with eventual loss of teeth (1). The severity of the disease is based both on the specific organisms isolated from infected sites, as well as the immune status of the host. The host response to periodontopathic organisms plays a major role in the outcome of periodontal diseases.

Porphyromonas gingivalis (*P. gingivalis*) has been implicated as a major etiological agent in the development of adult periodontitis due to its strong clinical correlation (1) and its ability to induce disease in primates(3). P. g is a highly adapted pathogen, which is armed with a number of putative virulence factors that enables this organism to cause disease. Among such virulence factors are fimbriae, a polysacharide capsule, lipopolysaccharide, hemagglutinating activities, outer membrane vesicles, and numerous enzymes (4-5).

P. gingivalis has been shown to advance into deeper epithelial layers *in vivo* (6-7), a process that could play a role in the spray of the organism. *P. gingivalis* has also been shown to bind and invade primary gingival epithelial cells, and multilayered pocket epithelial cells in *vitro* (8-10). Furthermore, Lamont et al. (8,9) have confirmed the ability of *P. gingivalis* to replicate within KB cells and within primary gingival epithelial cells.

Many epidemiological studies have strongly demonstrated the association of periodontal diseases with atherosclerosis and coronary heart disease (10, 11-13). Pathological studies have also identified *P. gingivalis* in diseased atherosclerotic tissue by PCR. In addition, *P. gingivalis* infection of *apoE* mice has been demonstrated to increase the mean area and the extent of atherosclerotic lesions histologically relative to those in unifected animals (14).

The ability of *P. gingivalis* to advance into deeper epithelial layers (6,7) could play a role in the systemic spread of the organism. The connective tissues of the periodontium are extremely well vascularized (2), allowing the invading microorganisms to readily enter the bloodstream. Thus, in addition to invasion of epithelial cells, the ability of *P. gingivalis* to actively invade endothelial cells could represent an additional mechanism evolved by this pathogen to evade the host response. Recent report by Genco et al. had showed the ability of *P. gingivalis* to invade and replicating in endothelial cells (15), indicating that this pathogen has the capability of localizing to the vascular wall (15, 16). Endothelial cells, among other vascular wall cells, may serve as reservoirs of *P. gingivalis* and *P. gingivalis* components and as contributors to immune stimulation during *P. gingivalis* infection. It has been proposed that *P. gingivalis* invasion of endothelial cells may induce alterations in the endothelial cells that could exhibit artherogenic properties (15, 16). However, it is not clear how active invasion of endothelial cells by *P. gingivalis* modulates the gene expression of endothelial cells. In this study, we used *P. gingivalis* 381 and its corresponding insertional mutant in the *fimA* gene (*P. gingivalis* DPG3) to infect HAEC, then analyze the altered gene expression in these infected HAEC by the GeneChip® Human Genome U95A (Affymetrix Inc.). The GeneChip® Human Genome U95A contains all full-length genes. This array

represents more than 12000 sequences previously characterized in terms of function or disease association. To further confirm the data obtained from oligonucleotide microarrays, we will choose several interesting genes that are significantly expressed in *P. gingivalis*-infected HAEC by RT-PCR

四、結果與討論

Microarray Analysis of *P. gingivalis* 381 infected HAEC

The transcriptional response of the human aortic endothelial cells to *P. gingivalis* 381 invasion was examined by hybridization of microarrays with biotin-labeled cRNA samples from uninfected HAEC or HAEC infected with *P. gingivalis* 381 for 1 hr or 6 hr. RNA message levels of 12633 genes were analyzed by HG-U95Av2 GeneChips (Affymetrix). *P. gingivalis* had been shown to adhere and invade into epithelial cells and endothelial cells over a period of 0-2 h . The intralcellular *P. gingivalis* 381 remain viable and may replicate inside the cells. Therefore, the relative transcript profiles were compared in a time-course experiment in which HAEC cells either briefly exposed to *P. gingivalis* 381 (1h) or incubated with bacteria for 6h. Since we were interested in defining the response of HAEC to extracellular *P. gingivalis* 381, for 6 hr incubation studies, extracellular *P. gingivalis* were washed following a 1h incubation period and the culture incubated for an additional 5h.

We observed the expression of 5591 and 5209 genes in unifected HAEC as assessing at 1h and 6 h, respectively. In *P. gingivalis* 381-infected HAEC at 6 h, we observed the upregulation of 69 genes which have at least 2 folds higher expression as compared to in uninfected HAEC, where 15 genes were induced from undetectable levels and 54 genes were upregulated when compared to uninfected HAEC. In *P. gingivalis* 381-infected HAEC at 1 h, upregulation of 13 genes was identified, where 1 gene were induced from undetectable levels and 12 genes were upregulated when compared to uninfected HAEC. From the data, we found the majority of genes expressed by HAEC under these experimental conditions were not affected by *P. gingivalis* infection, indicating that the endothelial cell mRNA response to *P. gingivalis* 381 infection was relatively narrow and specific.

Contribution of bacterial invasion to changes of HAEC gene expression

Since the adherence and invasion of *P. gingivalis* to endothelial cells depends on the expression of fimbriae, the transcript profiles in HAEC were compared from HAEC that were incubated with either wild-type or a major fimbriae knock-out mutant of *P. gingivalis* 381. This allowed us to determine if any of the differentially regulated genes identified in the time-couse experiment were the result of specific endothelial cell interactions with the *P. gingivalis* 381 major fimbriae. We identified 54 genes that had a 2-fold or greater increased expression in HAEC infected with wild-type *P. gingivalis* 381 when compared to those exposed to *fimA* mutant. Among these 54 genes, 26 genes were induced from undetectable level and 28 genes were upregulated when compared to *fimA*-infected HAEC. Only 4 genes had a 2-fold or greater increased expression in HAEC infected with wild-type *P. gingivalis* 381 also in HAEC infected with *fimA* mutant when compared to uninfected HAEC.

Confirmation of results obtained with Hu95av2 arrays by RT-PCR.

To confirm the data obtained using the human arrays and to further investigate the kinetics of the mRNA expression profiles for selected genes of interest, we choose a panel of inflammatory genes representing various chemokines whose expression levels were increased in *P. gingivalis*-infected HAEC. The mRNA expression levels of these selected genes were then analyzed by RT-PCR at various time points after *P. gingivalis* infection ranging from 0 to 24h. RNA samples from infected and uninfected HAEC were harvested and processed in parallel under identical conditions for each time point. As shown in Fig. 1 for the genes chosen for further analysis, RT-PCR confirmed the data obtained using the microarrays.

五、計畫成果自評

本研究計畫我們已完成下列之預期工作項目：

1. 建立 wild-type *P. gingivalis* 381 感染人類血管內皮細胞之後，血管內皮細胞基因表現之資料庫。

2. 建立纖毛基因剔除之 *P. gingivalis* DPG3 變異株感染人類血管內皮細胞之

後，血管內皮細胞基因表現之資料庫。

3. 藉由兩資料庫之比較建立 *P. gingivalis* fimbriae 調控血管內皮細胞基因表現之資料庫。

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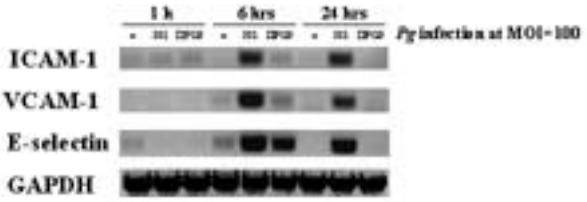
Figure 1. RT-PCR analysis of subsets of inflammatory gene expression in HAEC in response to *P. gingivalis* infection. *P. gingivalis* 381 (wt) or a *fimA* mutant were added to HAEC cultures at a MOI of 100:1, incubated at 37°C for 1 h, then culture supernatant were removed. After cells were washed 3 times with PBS, cells were incubatede in fresh culture media at 37°C for additional 5h, 23h. When total incubation period reached 6, 24h after *P. gingivalis* infection. HAEC were harvested and total RNA was extracted for mRNA expression analysis using RT-PCR. Control cultures were incubated with culture media only.



附件：封面格式

行政院國家科學委員會補助專題研究計畫成果報告

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※ 成人型牙周炎致病菌(Porphyromonas gingivalis)調控人類血管內皮細胞
※ 之基因表現之基因晶片研究。 ※
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計畫類別：☐個別型計畫 ☐整合型計畫
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共同主持人：賴銘堂 台北醫學大學耳鼻喉科
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