行政院國家科學委員會專題研究計畫成果報告

計畫編號：NSC 90-2314-B-038-039
執行期限：90 年 8 月 1 日至 91 年 7 月 31 日
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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。其主要致病菌之一 *P. gingivalis* 造成牙周炎的致病因子很多，其中蛋白質分解酵素(proteases)最引人注目。這些蛋白質分解酵素不僅能幫助 *P. gingivalis* 入侵宿主的牙齦組織及牙齦表皮細胞，甚至能將宿主的多項免疫防禦系統破壞。例如，將宿主的補體(complement) 或 Kallikrin/kinin 分解掉。最近的研究更進一步發現蛋白質分解酵素之一的 gingipain K (KGP)及 gingipain R (RGPA) 能將 tumor necrosis factor-a (TNF-α) 分解使其失去活性。Darveau 等人的研究也指出 *P. gingivalis* 侵入牙齦表皮細胞之後，會抑制 Interleukin-8 (IL-8)之表現量。由這些證據顯示 *P. gingivalis* 除了利用 KGP 及 RGP 侵入牙齦表皮細胞、牙齦組織表皮層及結締組織之外，其也能藉由調控宿主免疫細胞分泌之炎性或趨化性介質來控制宿主之免疫發炎反應。

在牙齦表皮層之下的結締組織內有許多血管，*P. gingivalis* 有可能藉由侵入血管內皮細胞進入血管系統造成宿主身體其它器官之疾病。最近，有許多研究指出成人型牙周炎病患罹患心血管疾病之機率高於未罹患牙周炎之民眾。另外，Genco 等人的研究也指出 *P. gingivalis* 能侵入人類之血管內皮細胞。血管內皮細胞分泌趨化性介質(如 IL-8)使免疫細胞聚集於細菌感染部位為宿主抵抗細菌感染之早期防線。因此。本研究之目的在於探討 *P. gingivalis* 之蛋白質分解酵素中活性及作用最強的 KGP 及 RGP 對人類血管內皮細胞感染 *P. gingivalis* 之後分泌或表現趨化性介質 IL-8 及 MCP-1 之調控機轉。

Keywords: *Porphyromonas gingivalis*, protease inhibitor, RNase protection Assay, IL-8, MCP-1.

二、英文摘要

Adult periodontitis is a chronic inflammatory disease that is caused by a mix infection of bacteria. *Porphyromonas gingivalis* (*P. gingivalis*) has been implicated as a major etiological agent in the development of adult periodontitis due to its strong clinical correlation and its ability to induce disease in primates. P. g is a highly invasive pathogen which is armed with two potent proteinases which are referred to as Arg-gingipain (RGPA) and Lys-gingipain (KGP), respectively. Significantly, these enzymes can inactivate many of the host plasma proteinase inhibitors, essentially dysregulating each of the cascade of complement, Kallikrein/kinin, and coagulation/ fibrinolytic pathways. In addition, KGP and RGPA had been reported to inactivate tumor necrosis factor-α, the first line cytokine in innate host defense system, in vitro. Because of their role in invasion into host tissue and cells, and their roles in modulating host immune system,

Kgp and Rgp have raised much attention in recent studies of *P. gingivalis* associated with varieties of host cells.

Periodontal diseases have recently been associated with cardiovascular disease and preterm delivery of low birth weight infants. The connective tissues of the periodontium are extremely well vascularized. Thus, the ability of *P. gingivalis* to invade into epithelial layers and endothelial cells could play a role in the systemic spread of the organisms.

For a number of bacteria entry into host cells elicits cytokine production, inducing secretion of IL-1, IL-6, IL-8 and MCP-1 that leading to recruitment of leukocytes. However, in recent report by Madianoset al. demonstrated that the invasion of *P. gingivalis* into a epithelial cells line, attenuated the production of IL-8. Darveau and colleagues have also reported that *P. gingivalis* invasion of gingival epithelial cells inhibits IL-8 accumulation. From these evidences, it is interesting to speculate that the *P. gingivalis* cysteine proteinases (KGP or RGPA) could function in some capacity in the degradation of these molecules. Hypothetically, the P. g infection with HUVEC might also have effects on the synthesis of chemotatic cytokines such as IL-8 and MCP-1 in HUVEC.

To test the hypothesis, we infected HUVEC with *P. gingivalis* or KGP or RGPA mutant of *P. gingivalis*. In addition, *P. gingivalis* pretreated with proteases inhibitors specific for KGP or RGP were used to investigate the role of gingipain R or K effect on IL-8 and MCP-1 synthsis in HUVEC.

Keywords: *Porphyromonas gingivalis*, protease inhibitor, RNase protection Assay, IL-8, MCP-1.

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).

Porphyromonas gingivalis (*P. gingivalis*) has been implicated as a major etiological agent in the development of adult periodontitis due to its strong clinical correlation (1-3). P. g is a highly adapted pathogen which is armed with a number of putative virulence factors which enables this organism to cause disease (4-5). Among such virulence factors, two members of a family of cysteine proteases synthesized and secreted by *P. gingivalis* play critical parts in interactions with host cells. The two proteinases are referred to as Arg-gingipain (RGP) and Lys-gingipain (KGP), because of their specificity for cleavage after arginyl and lysyl residues, respectively. Significantly, these enzymes can inactivate many of the host plasma proteinase inhibitors, essentially dysregulating each of the cascade of complement, Kallikrein/kinin, and coagulation/ fibrinolytic pathways (6-11). In addition, KGP and RGP had been reported to inactivate tumor necrosis factor-α(12), the first line cytokine to launch inflammatory responses in host. Because of their role in invasion into host tissue and cells, and their roles in modulating host immune system, Kgp and Rgp have raised much attention in recent studies of *P. gingivalis* associated with varieties of host cells. The invasion ability of P. g to epithelial cells was significantly inhibited when *P. gingivalis* were pre-treated with proteases inhibitors specific to KGP and RGP(18). Periodontal diseases have recently been associated with cardiovascular disease and preterm delivery of low birth weight infants (17, 19-21). The ability of *P. gingivalis* to advance into deeper epithelial layers (13-16) could play a role in the systemic spread of the organism. Recent report by Genco et al. had showed the ability of *P. gingivalis* to invade bovine aortic endothelial cells, and human umbilical vein endothelial cells (HUVEC)(22).

For a number of bacteria entry into host cells elicits cytokine production, inducing proinflammatory responses through

secretion of IL-1, IL-6, IL-8 and MCP-1 that leading to recruitment of leukocytes (23). However, in recent report by Madianoset al. demonstrated that the invasion of *P. gingivalis* into a epithelial cells line, attenuated the production of IL-8 (24). Darveau and colleagues (25) have also reported that *P. gingivalis* invasion of gingival epithelial cells inhibits IL-8 accumulation. From these evidences, it is interesting to speculate that the *P. gingivalis* cysteine proteinases (KGP or RGP) could function in some capacity in the degradation of these molecules. Hypothetically, the *P. gingivalis* infection with HUVEC might also have effects on the synthesis of chemotatic cytokines such as IL-8 and MCP-1 in HUVEC.

In this study, HUVEC were infected with *P. gingivalis* or *P. gingivalis* mutant of proteinases KGP or RGP. In addition, *P. gingivalis* pretreated with proteases inhibitors specific for KGP or RGP were also used to infect HUVEC to investigate the role of gingipain R or K effect on IL-8 and MCP-1 synthsis in HUVEC. The synthesis of IL-8 and MCP-1 in HUVEC infected with *P. gingivalis* were analyzed at protein level by ELISA and at RNA level by RNase protection assay.

四、結果與討論

In this study we examined the consequences of *P. gingivalis* infection of HUVEC on the expression of the chemokines IL-8 and MCP-1 response. HUVEC constitutively produced low levels of IL-8 (Fig. 1). Coculture of HUVEC with live *P. gingivalis* A7436 abolished the IL-8 and MCP-1 responses (Fig.1). Pretreatment of *P. gingivalis* with protease inhibitors prior to the addition to HUVEC prevented the inhibition of IL-8 transcription and protein expression in *P. gingivalis*-infected HUVEC, indicating that the inhibition was proteolytically mediated (Fig.2-3). Coculture of HUVEC with a *P. gingivalis* mutant deficient in lysine-speicific cystein proteinases (gingipainK[KGP]) or arginine -speicific cystein proteinases (gingipainK[KGP]) resulted in an increase in both IL-8 and MCP-1 production relative to that observed in HUVEC cocultured with the *P. gingivalis* wild-type strain 33277 (Fig.2-3). Analysis of *P. gingivalis* infected HUVEC cultures by an RNase protection assay revealed an increase in the IL-8 transcript relative to uninfected HUVEC (Fig.4). These results indicate that *P. gingivalis* can temporally modulate the chemokine response in endothelial cells through gingipain-mediated mechanisms.

Figures and Legends.

Fig.1 Pretreatment of *P. gingivalis* with protease inhibitors stimulates IL-8 in *P. gingivalis*-infected endothelial cells. *P. gingivalis* A 7436 cultures were incubated wit protease inhibitors for 1 h at 37°C under anaerobic conditions, washed, and resuspended in HUVEC growth media. HUVEC monolayers were infected with 1.0 ml of the bacterial suspension (MOI of 1:100) and incubated at 37°C in 5% CO_2 for 16 and 24 h. Supernatant samples were collected and analyzed by ELISA for IL-8. Gray bars, HUVEC infected with *P. gingivalis* preincubated with ZFKck; stippled bars, HUVEC infected with *P. gingivalis* preincubated with leupeptin; horizontal bars, HUVEC infected with *P. gingivalis* preincubated with protease inhibitor cocktail; black bars, uninfected HUVEC; open bars, HUVEC pretreated with dH_2O. The data are expressed relative to the IL-8 expressed by HUVEC infected with *P. gingivalis* preincubated with dH_2O, and are the means $\pm$ standard deviations for at least two separate experiments performed in duplicate. *, p value of <0.1 compared to a control culture of *P. gingivalis* preincubated with dH_2O only.

Fig.2 *P. gingivalis* infection of endothelial cells stimulates IL-8 transcription. *P. gingivalis* A 7436 cultures were incubated wit protease inhibitors for 1 h at 37°C under anaerobic conditions, washed, and resuspended in HUVEC growth media. HUVEC monolayers were infected with 1.0

ml of the bacterial suspension (MOI of 1:100) and incubated at 37°C in 5% CO_2 for 24 h. Then the RNA was extracted. Two micrograms of total RNA was hybridized to the probe template set. Nuclease-protected RNA fragments were analyzed on a polyacrylamide gel, which was subsequently exposed to Imaging Screen-K. The band intesities were determined with Quantity-one software, and the mRNA expression levels for IL-8 were normalized with respect to the average intensities of the bands of the housekeeping genes (HKG) GAPDH and L32. Black bars, uninfected HUVEC; open bars, HUVEC infected with *P. gingivalis*; gray bars, HUVEC infected with *P. gingivalis* preincubated with ZFKck; stippled bars, HUVEC infected with *P. gingivalis* preincubated with leupeptin; horizontal bars, HUVEC infected with *P. gingivalis* preincubated with protease inhibitor cocktail. The data are the means ± standard deviations for at least two separate experiments performed in duplicate. *, p value of <0.2 compared to unifected HUVEC.

Fig.3 Infection of endothelial cells with *P. gingivalis kgp* and *rgpA* mutants stimulate IL-8 and MCP-1 production. *P. gingivalis* strain 33277 or the corresponding *kgp* (YPP1) and *rgpA* (YPP2) mutants was added to the HUVEC monolayer at a MOI of 1:100 and incubated at 37°C in 5% CO_2 for 16h, 24h and 48h. . At designated time point, the supernatant samples were collected and analyzed by ELISA for IL-8 (A) and MCP-1(B). Black bars, uninfected HUVEC; gray bars, HUVEC infected with *P. gingivalis* 33277; open bars, HUVEC infected with *P. gingivalis* YPP1; horizontal bars, HUVEC infected with *P. gingivalis* YPP2. The data are the means ± standard deviations for at least two separate experiments performed in duplicate. *, p value of <0.05 compared to unifected HUVEC cultures challenged with the wild-type strain *P. gingivalis* 33277.

Fig.4 Infection of endothelial cells with a *P. gingivalis kgp* mutant upregulate IL-8 transcription. *P. gingivalis* 33277 (wt) or YPP2 *(kgp)* mutant were added to the HUVEC monolayer at a MOI of 1:100 and incubated at 37°C in 5% CO_2. Control, uninfected HUVEC. Samples were removed at 24 h postinfection, and RNA was extracted from HUVEC. Two micrograms of total RNA was hybridized to the probe template set. Nuclease-protected RNA fragments were analyzed on a polyacrylamide gel, which was subsequently exposed to Imaging Screen-K. The band intesities were determined with Quantity-one software, and the mRNA expression levels for IL-8 were normalized with respect to the average intensities of the bands of the housekeeping genes (HKG) GAPDH and L32. The data are the means ± standard deviations for at least two separate experiments performed in duplicate. *, p value of <0.05 compared to unifected HUVEC cultures challenged with the wild-type strain *P. gingivalis* 33277.

Fig.1

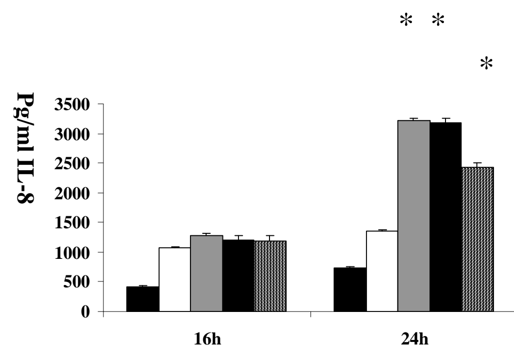
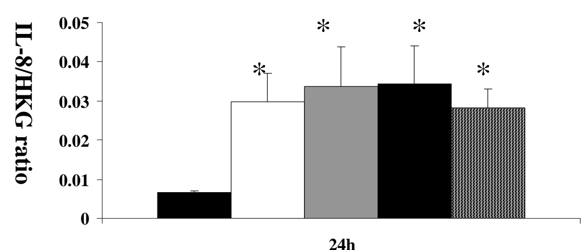


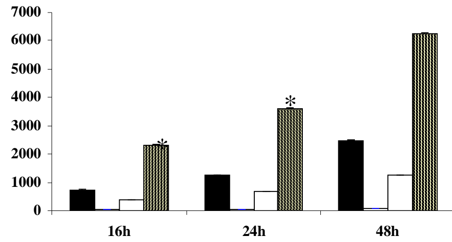
Fig. 2



Pg/ml IL--8

Fig.3

A



B

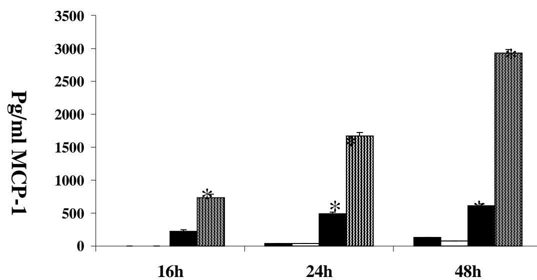
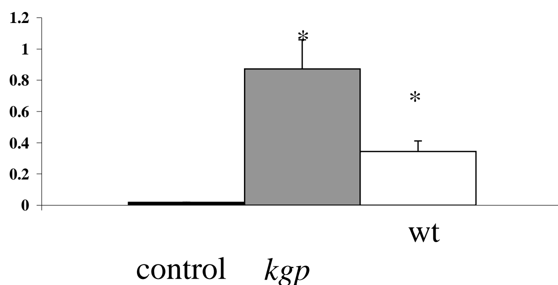


Fig.4



control *kgp*

五、計畫成果自評

此研究的結果顯示 *P. gingivalis* 之蛋白質分解酵素KGP及RGPA 會分解血管內皮細胞分泌的趨化性介質。此研究的結果可以增進我們了解 *P. gingivalis* 在牙周炎致病機轉中可能扮演的角色，並提供我們一個新方向去思考如何藉由調控 *P. gingivalis* 的致病因子來控制牙齦組織中由 *P. gingivalis* 所引起之一連串免疫反應及組織的破壞，進而控制牙周炎之病程進展。

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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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※　人類心臟血管內皮細胞分泌趨化性介質之作用機轉　※
※　之體外研究　　　　　　　　　　　　　　　　　　※
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計畫類別：☒個別型計畫　　☐整合型計畫

計畫編號：NSC 90-2314-B-038-039

執行期間：　90 年 8 月 1 日至 91 年 7 月 31 日

計畫主持人：周幸華　台北醫學大學牙醫學系

共同主持人：賴銘堂　台北醫學大學耳鼻喉科

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☐出席國際學術會議心得報告及發表之論文各一份

☐國際合作研究計畫國外研究報告書一份

執行單位：

中　華　民　國　　　年　　　月　　　日