Virulence of Cultured Supernatant in *Porphyromonas gingivalis* W50 under Hemin- and Menadione-Limited Culture Condition

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I. Introduction

Porphyromonas gingivalis(P. gingivalis) has been isolated frequently and in high numbers from periodontal pockets of adult patients with advanced periodontitis, while the organism is recovered rarely and only in low numbers from healthy gingival sites[1-3]. Many virulence determinants of *P. gingivalis* are cell surface components or extracellular products, such as capsules, toxins, and enzymes, and their synthesis and secretion can be markedly affected by growth conditions[3]. In the host, the growth of pathogens is at submaximal rates when the essential nutrients or cofactors are at growth-limiting concentrations. Indeed, it has been argued that some virulence determinants might only be expressed *in vivo*, due to specific growth conditions, while potential factors identified in the laboratory may play little or no role in disease[4]. For studying virulence factors of pathogens in the laboratory, it is important to take into account the growth conditions that exists in the host.

Even though the precise environmental conditions at any one time in a periodontal pocket or in an animal model during the course of an infection are not known, it has been shown recently that growth of a number of pathogenic Gram-negative bacteria either in chambers implanted in animals or at sites of infection in humans is either iron limited or severely restricted by the availability of iron. Thus, the ability to compete for iron with the host will contribute significantly to the virulence of an organism[3), 5-6].

The black-pigmented *Bacteroides* spp. which includes *P. gingivalis* cannot utilize free iron, but they do have a requirement for heme. It is signigicant that *P. gingivalis* has been found to be most effective at degrading the iron-transporting plasma proteins albumin,

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hemopexin, haptoglobin, and transferrin[7]. Because of the apparent key role of hemin or menadione in the growth of bacteria in the host, cells were grown in a medium containing hemin or menadione. *P. gingivalis* is an strict anaerobe with fastidious growth requirements such as both hemin and menadione, and produces volatile and nonvolatile metabolic acids, including butyric acid, propionic acid, valeric acid, and isobutyric acid. It was reported that cells grown under hemin-limited conditions had a reduced virulence in mice compared with bacteria cultured in an excess of the cofactor, and the decreased transport of hemin results in the increased expression of several virulence factors which may be regulated by hemin[8-9]. Any change in growth conditions of a particular microhabitat may affect the metabolic expression and the pathogenic potential of some species, i.e. *S. mutans, S. sanguis*, and *A. viscosus*[10]. Recently, the volatile fatty acids produced by periodontopathic bacteria were reported to easily penetrate the oral mucosa, causing severely harmed periodontal tissue including immunoregulatory cells[11]. The purpose of this study was to investigate *in vivo* virulence of cultured supernatant under both hemin- and menadione-limited culture condition in *P. gingivalis*.

II. Materials and Methods

1. Bacterial cultures

P. gingivalis W50, an invasive strain, was cultured anaerobically(80% N_2, 10% CO_2, 10% H_2) for 48h at 37℃ in peptone-yeast extract-glucose(PYG) broth. After purity check by Gram stain and aerobic and anaerobic blood agar plate cultures, the broth supernatants were recovered. *P. gingivalis* W50 was also cultured anaerobically under both hemin- and menadione-limited culture condition(80% N_2, 10% CO_2, 10% H_2) for 48h at 37℃ in peptone-yeast extract-glucose (PYG) broth.

2. *In vitro* virulence test

The virulence of cells under hemin- and menadione-limited culture condition was compared *in vitro*. Fresh cells were concentrated by centrifugation(10,000 g for 10 min) and suspended in PYG broth to adjust optical density. NIH 3T3 cells were grown in Eagle minimal essential medium containing 5% fetal bovine serum (heat inactivated), 100 IU/㎖ of penicillin and 100㎍/㎖ streptomycin. The cells were incubated at 37℃ in humidified air containing 5% CO_2. The cell growth and proliferation assay is dependent on the cellular reduction of methylthizol-2-yl-2, 5-diphenyl tetrazolium bromide(MTT, Sigma Chemical CO., St Louis, MO) by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically[12].

Following 24 hrs incubation of cells at 37℃ for a further four hours before processing, plates were then processed as described[12] and absorbance was measured at 570㎚ using a ELISA analyser(Model ETY-96, Toyo instruments Inc., Japan). The cell activity was determined by the formula: [mean absorbance in twelve test wells/mean absorbance in twelve control wells] X 100.

3. Prepartion for mouse pathogenicity test

The virulence of cells under both hemin- and menadione-limited culture condition was compared in mice. Fresh cells were concentrated by centrifugation(10,000 × g for 10 min) and suspended in PYG broth to adjust optical density. Female ICR mice (body weight 20 to 25 g) were anesthetized with ether and injected subcutaneously at two sites 1㎝ lateral to the midline on the dorsal surface, with 0.1㎖ of the bacterial culture supernatant standardized to give approximately 1×10^9 cells/㎖. Postmortem examination was carried out with samples of the injection sites. Samples were fixed in 10%(vol/vol) neutral buffered formalin.

4. Histological study

Blocks of formalin-fixed tissues were processed by standard procedures and embedded in paraffin wax. The sections were cut at 5㎛ and stained with hematoxylin and eosin(H & E). Mice injected with PYG broth were used as controls for the histologic studies to assess the inflammatory response in the inoculum.

5. Statistics

Using the ANOVA, the statistical significance of the differences among hemin or menadione treated groups was determined.

III. Results

In the study of NIH 3T3 cell activity of cultured supernatant of *P. gingivalis* W50, the cell activity in the presence of hemin was lower than that in the presence of PBS, *P. gingivalis* W50 in the absence of hemin and menadione, *P. gingivalis* W50 in the presence of menadione, and *P. gingivalis* W50 in the presence of hemin and menadione.

In the clinical course of P. gingivalis W50 infection under limited culture condition in mice, interstitial inflammatory cell infiltration was rare in PYG broth group(Fig. 1 and Table 1). Interstitial edema and muscular destruction were moderate, inflammatory cell infiltration and hemorrhage or congestion were moderate in *P. gingivalis* W50 in the absence of hemin and menadione(Fig. 2 and Table 1). Hemorrhage or congestion was moderate, and interstitial edema, inflammatory cell infiltration and muscular destruction were mild in the presence of hemin(Fig. 3 and Table 1), as well as in the presence of menadione(Fig. 4 and Table 1). However, inflammatory cell infiltration was severe, and interstitial edema, hemorrhage or congestion, and muscular destruction were moderate in the presence of hemin and menadione(Fig. 5 and Table 1).

Table 1 ***In vitro*** **virulence test of culture supernatant of** ***P. gingivalis*** **W50 under hemin- and menadione-limited culture condition**

	Cell activity(%)
PBS	100.29±8.95
P. gingivalis W50	100.94±10.42
Hemin	81.47±5.44*
Menadione	100.77±9.40
Hemin and Menadione	100.74±7.50

*Statistically significant compared to PBS, *P. gingivalis* W50 in the absence of both hemin and menadione, *P. gingivalis* W50 in the presence of menadione, and *P. gingivalis* W50 in the presence of both hemin and menadione.

Table 2 In vivo virulence test of culture supernatant of *P. gingivalis* under hemin- and menadione-limited culture condition

	Interstitial edema	Inflammatory cell infiltration	Hemorrhage or congestion	Muscular destruction
PBS	+/-	+/-	+/-	+/-
P. gingivalis W50	++	++	++	+
Hemin	+	+	++	+
Menadione	+	+	+	+
Hemin and Menadione	++	+++	++	++

+/-, rare; +, mild; ++, moderate; +++, severe.

IV. Discussion

Bacterial virulence depends on the invasiveness and toxigenicity of the strain. The virulence of *P. gingivalis* W50 infection under limited culture condition in mice, inflammatory cell infiltration was severe only in the presence of hemin and menadione, but mild under hemin- or menadione-limited culture conditions, suggesting that the virulence of *P. gingivalis* may be related to nutritional conditions.

Even in the absence of hemin and menadione, interstitial inflammatory cell infiltration was rare in PYG broth. Inflammatory cell infiltration and hemorrhage or congestion was moderate, interstitial edema and muscular destruction were mild in *P. gingivalis* W50 in the absence of hemin and menadione. These data suggest that *P. gingivalis* growth in the absence of exogenous menadione, and the stored both hemin and menadione may be utilized under limited culture condition.

The increased virulence in the absence of hemin might be related to the increased membrane vesicles, hemolytic and trypsin-like protease activities, seen in a Tn4351-generated hemin uptake mutant of *P. gingivalis*[9]. However, the virulence of *P. gingivalis* W50 grown under conditions without any added hemin was lower than that grown found in the presence of hemin[8].

In our experiment, the cell activity in the presence of hemin was lower than that in the absence of hemin and menadione. Hemorrhage or congestion was moderate, and interstitial edema, inflammatory cell infiltration, and muscular destruction were mild in the presence of hemin. Hemin might be related to the expression of virulence factor such as fimbriae[8].

Inflammatory cell infiltration, interstitial edema, hemorrhage or congestion, and muscular destruction were mild in the presence of menadione. Our previous experiment reported that the virulence of *P. gingivalis* W50 in the presence of menadione was far more severe than that of hemin[13].

The virulence of *P. gingivalis* W50 in the presence of hemin was increased by the secretion of virulence factor more than that of menadione, and inflammatory cell infiltration was severe, and interstitial edema, hemorrhage or congestion, and muscular destruction were moderate in the presence of hemin and

menadione.

These results suggest that hemin increase the secretion of virulence factor in *P. gingivalis* W50 through extracellular vesicle[9]. All of these results suggest that the virulence of cultured supernatant in *P. gingivalis* W50 might be affected by both hemin and menadione.

Further studies are needed to evaluate continuous culture devices for *P. gingivalis* and to monitor the change of virulence under excessive hemin and menadione conditions.

V. Conclusion

To investigate the change of virulence in *P. gingivalis* W50 under hemin- and menadione-limited culture condition, *in vitro* virulence was measured by the change of NIH 3T3 cell activity and *in vivo* virulence was measured by the inflammatory change of subcutaneous tissue in ICR mouse.

The mouse 3T3 cell activity of cultured supernatant of *P. gingivalis* W50 in the presence of hemin was lower than that in the absence of hemin and menadione. The inflammatory cell infiltration and hemorrhage or congestion were moderate, interstitial edema and muscular destruction were mild in the absence of hemin and menadione. Also, in the presence of menadione, inflammatory cell infiltration, interstitial edema, hemorrhage or congestion, and muscular destruction were mild. Hemorrhage or congestion was moderate, and interstitial edema, inflammatory cell infiltration and muscular destruction were mild in the presence of hemin. Inflammatory cell infiltration was severe, and interstitial edema, hemorrhage or congestion, and muscular destruction were moderate in the presence of hemin and menadione.

These results suggest that the virulence of cultured supernatant in *P. gingivalis* W50 might be affected by hemin.

VI. Acknowledgements

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VII. References

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사진부도 설명

Fig 1 Histopathological features of PYG broth. Interstitial inflammatory cell(arrow) infiltration was rare (Hematoxyline & Eosin).

Fig 2 Histopathological features of *P. gingivalis* W50 group. Interstitial edema, inflammatory cell (arrow) infiltration, and hemorrhage or congestion was moderate. Muscular destruction was mild(Hematoxyline & Eosin).

Fig 3 Histopathological features of hemin added group. Hemorrhage(arrow) or congestion was moderate. Interstitial edema and inflammatory cell infiltration and muscular destruction was mild(Hematoxyline & Eosin).

Fig 4 Histopathological features of menadione added group. Inflammatory cell infiltration, interstitial edema, hemorrhage or congestion(arrow) and muscular destruction was mild (Hematoxyline & Eosin).

Fig 5 Histopathological features of both hemin and menadione added group. Inflammatory cell(arrow) infiltration was severe. Interstitial edema, hemorrhage or congestion and muscular destruction was moderate(Hematoxyline & Eosin).

사진 부도(I)

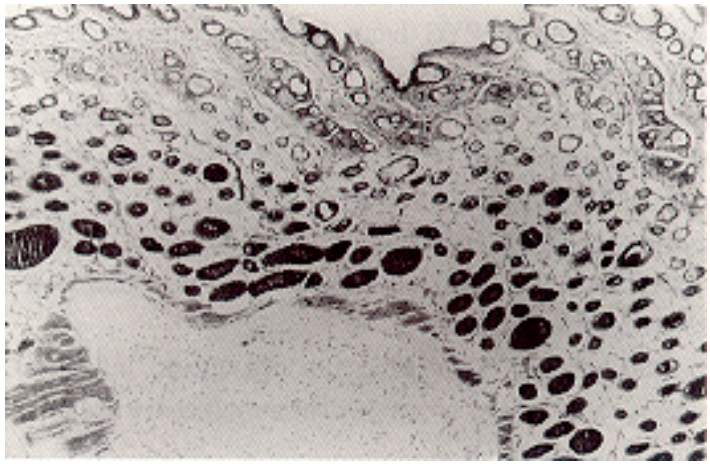

Fig 1

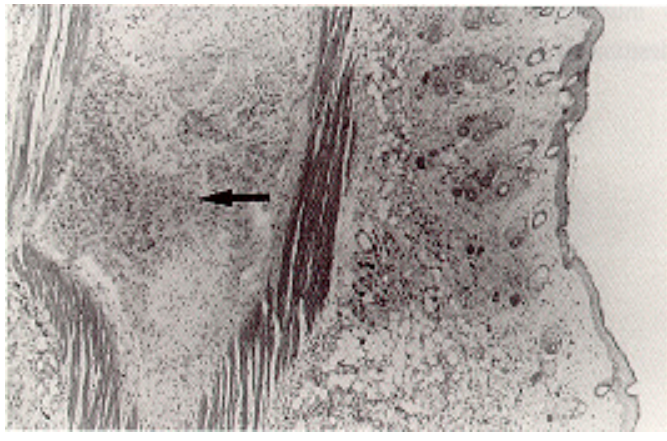

Fig 2

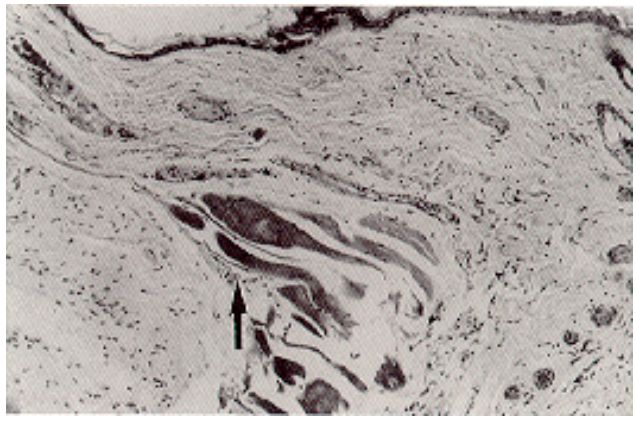

Fig 3

사진 부도(Ⅱ)

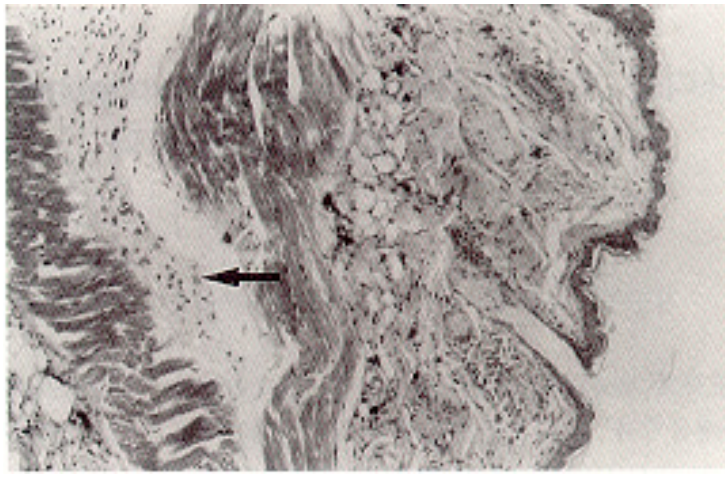

Fig 4

Fig 5

국문초록

헤민과 메나디온 제한 조건에서 배양한 Porphyromonas gingivalis W50의 배양 상청액의 병독력

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헤민과 메나디온 제한에 의한 Porphyromonas gingivalis(P. gingivalis) W50의 병독력의 변화를 검색하고자, 실험관내 병독력을 NIH 3T3 세포의 세포활성 변화로 관찰하였고, 생체내 병독력은 배양상청액을 ICR mouse 피하조직에 주사한 후의 염증반응을 관찰하였다.

헤민 존재 하에 배양한 P. gingivalis W50 배양상청액에 의한 mouse 3T3 세포의 세포활성은 헤민과 메나디온 없이 배양한 세포의 활성보다 낮았다.

헤민과 메나디온을 첨가하지 않고 배양한 세균의 생체내 병독력은 중등도의 염증세포 침윤과 울혈에 의한 출혈, 미약한 세포간질의 부종과 근육 파괴를 보였다. 메나디온 존재 하에서 배양한 세균은 미약한 염증세포의 침윤, 울혈에 의한 출혈 및 근육의 파괴가 관찰되었다. 헤민 존재 하에서 중등도의 울혈에 의한 출혈, 미약한 세포간질의 부종, 염증세포의 침윤 및 근육파괴가 관찰되었다. 헤민과 메나디온 존재 하에서 배양한 세균은 심한 염증세포의 침윤과 중등도의 세포간질의 부종 및 울혈에 의한 출혈을 보였다.

이상의 연구 결과 P. gingivalis W50 배양 상층액의 병독력은 헤민에 의하여 영향을 받는 것으로 생각된다.

주요어: 헤민, 메나디온, 병독력, 세포활성