Intracellular Invasion of *Staphylococcus aureus* against Human Gingival Fibroblasts

Kang-Ju Kim[1], Kyu-Yong Jung[2]

[1]Department of Oral Microbiology, College of Dentistry, Wonkwang University, Iksan, Jeonbuk, South Korea
[2]Department of Pharmacology, College of Medicine, Wonkwang University, Iksan, Jeonbuk , South Korea

I. Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common bacterial pathogens, causing approximately 80% of all cases of osteomyelitis with a broad host range in coagulase-positive staphylococci and is one of the leading causes of infections in oral and maxillofacial regions[1, 2]. Moreover, the rapidly increasing frequency of antibiotic-resistant bacteria has caused considerable alarm within the dental and medical community[3, 4]. Infections associated with this organism are extremely common and often life threatening; therefore, there is serious potential for *S. aureus* to cause increased morbidity and mortality in oral and maxillofacial regions[3-6].

To cause the infection, the bacteria do the followings: adhere and enter the host, multiply on or in the host tissues, resist host defences, and damage the host. All four processes must be accomplished in order to produce an infectious disease, and each is complex, involving several determinants. Bacterial attachment and penetration into underlying tissues play an important role in the initiation of infection. Some bacteria may penetrate tissue surfaces by producing lytic substances that break down the tissue surface, easing penetration to the deeper tissues, and other bacteria can penetrate through epithelial cells. It is likely that a variety of mechanisms are used by different organisms to penetrate the various tissue surfaces that they use as their portal of entry[7]. However lately, many authors have demonstrated the capability of the bacteria to attach on and invade the internal space of non-phagocytic eukaryotes[8-13]. On the other hand, it has been suggested that the continuous process of actin filament elongation provides the driving force for bacterial propulsion in infected cells or cytoplasmic extracts[14-16]. Several unrelated intracellular bacterial pathogens, including *Listeria monocytogenes* (*L. monocytogenes*), *Shigella flexneri, Rickettsiae* and Vaccinia virus, are known to share the ability to use actin polymerization as a driving force for intracellular movement, cell-to-cell spread and dissemination within infected tissues.

While *S. aureus* has not traditionally been considered to be an intracellular pathogen, previous studies have revealed that *S. aureus* may be actively internalized by phagocytosis and are capable of intracellular survival in epithelial and endothelial cells[17, 18]. It has also been suggested that *S. aureus* interact with or binds to the cell surface of bovine

mammary epithelial cells and is able to invade and appear free in the cytoplasm of cultured chicken osteoblasts[19]. However, little is known about the mechanism involved in the internalization of *S. aureus* by the host cells in oral infection.

Nevertheless, little is known regarding the mechanism involved in the characteristics of internalization, the potential role of internalization of *S. aureus* by the host cell, or host cell responses. In our previous study[20], the interaction of staphylococcal protein A and cytoskeletal actin filaments plays a pivotal role in the *S. aureus* invasion to cultured KB cells, established human epidermoid carcinoma cells. In expansion of our previous study, our present study was designed to certify whether *S. aureus* can invade the internal space of human gingival fibroblasts (HGFs) and make clear the difference between *S. aureus* ATCC 25923 and the wild type OPT 2 invasion of HGFs. This study was also designed to investigate whether *S. aureus* can invade HGFs via cytoskeletal actin proteins.

II. Materials and Methods

1. Bacteria strain and growth conditions

S. aureus ATCC 25923 was purchased commercially and a wild type strain of *Staphylococci aureus* (OPT 2) was sampled from the patients with acute oral and maxillofacial infections. *S. aureus* Wood 46 was kindly supplied by Dr. Hideo Igarashi (Tokyo Metropolitan Research Laboratory for Public Health). Before each experiment, a single colony from a blood agar plate (Difco Laboratories, Detroit, MI, USA) was inoculated into brain heart infusion (BHI, Difco Laboratories) broth and grown overnight at 37℃ with vigorous shaking. An 100 μl of the culture was transferred into 10 ml of BHI broth and incubated for 8 hrs at 37℃ with vigorous shaking. The culture was centrifuged and the pellet was washed once with sterile phosphate-buffered saline (PBS, pH 7.4), and resuspended in the tissue culture medium (without antibiotics; see below) to give a cell density of 10^5cfu/ml unless otherwise specified.

2. Cell culture

HGFs were obtained from the patients undergoing oral sugery. After 3 or 5 passages, the cells were used in this study. The HGFs were grown routinely in monolayers in Eagle's minimum essential medium (α-MEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G and 10 μg/ml streptomycin sulphate. Before use, cells were seeded at 5×10^4 cells/well in 24-well tissue culture plates (Costar, Cambridge, MA, USA) for binding and invasion assays and grown into confluent monolayers for 1 day in air at 37℃ with 5% CO_2.

3. Invasion assay

To investigate the bacterial invasion to cultured monolayers of HGFs, the previous methods were used with a slight modification[21, 22]. Approximately 16 hrs prior to the experiments, HGFs were washed three times with invasion medium (growth medium without antibiotics) and held in this medium. Just before beginning the experiment, the medium was removed and the HGFs were washed once with invasion medium followed by a further addition of 1 ml of fresh invasion medium. Appropriate wells of HGFs were inoculated with 1 ml of invasion medium containing 10^5cfu/ml/well (or other doses) of bacteria for the specified times at 37℃ in air with 5% CO_2. Subsequently, the medium was removed from the infected monolayers, before washing three times with sterile Ca^{2+}- and Mg^{2+}-free PBS to remove non-

adherent bacteria. The HGFs were treated with 0.25% trypsin in Hanks' balanced salt solution (Gibco BRL) and further lysed with 0.025% Triton X-100 (Sigma, St. Louis, MO, USA) in sterile distilled water. Cell lysates were serially diluted 20-fold and plated in triplicate on blood agar plates; the plates were then incubated overnight at 37℃ and the Colony-Forming Units (cfu) were counted. At this time, colonies of *S. aureus* were identified by Gram stain, catalase and coagulase tests. To quantify membrane-adherent and intracellular bacteria, gentamicin was used to kill any extracellular bacteria. After the HGFs had been incubated with bacteria for the specified times at 37℃ in air with 5% CO_2, super natants were removed and replaced with 1 ml of medium containing of 10 μg/ml gentamicin (Gibco BRL), followed by incubation at 37℃ with 5% CO_2. After 2 hrs, the supernatants were removed and discarded. The HGFs were washed three times with sterile PBS, and intracellular bacteria were recovered and counted as described previously.

4. Treatment with CB and CD

To test the inhibition of bacterial invasion by CB and CD, an invasion assay was done as described above, in the presence or absence of CB and CD (Sigma). HGFs were incubated with normal growth medium containing CB and CD at a given concentration for 30min at 37℃ in air with 5% CO_2, followed by incubation of the HGFs with *S. aureus* (1×10^5 cfu/ml) in the presence of the same concentration of CB and CD.

III. Results

1. Effect of cell numbers and time on *S. aureus* invasion to HGFs

In order to measure the efficiency of *S. aureus* ATCC 25923 and OPT 2 infection to HGFs, the

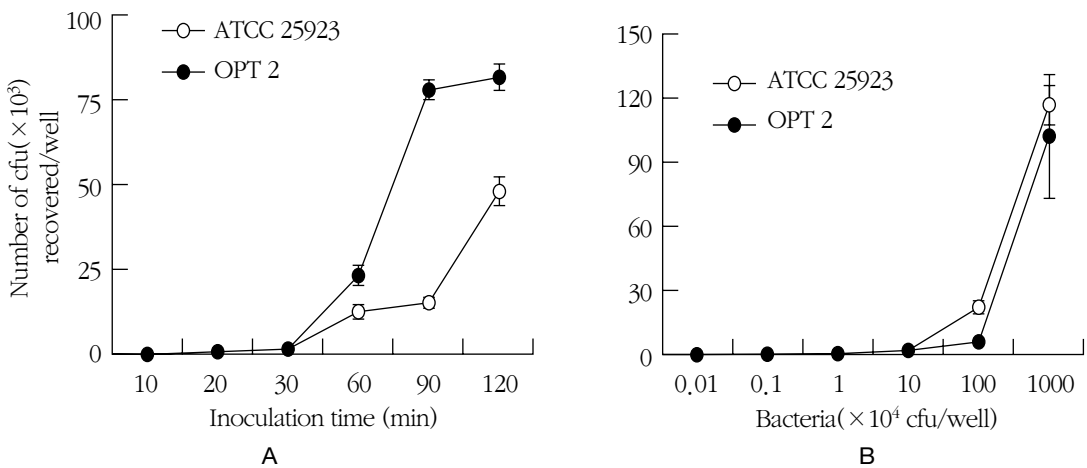

Figure 1. Kinetics of *S. aureus* ATCC 25923 and OPT 2 infection of HGFs. Monolayers were incubated with 10^5cfu/ml for the specified times (A) or different numbers of bacteria for 30 min (B), followed by measurement of colony formining unit (cfu) recovered from cell monolayers. Values represent the means ±SE of 8 to 10 determinations with triplicate. Longer incubation time than 60 min resulted in an increase in the number of cfu recovered (A). An increase in the inoculum size resulted in an increase in the number of cfu recovered (B).

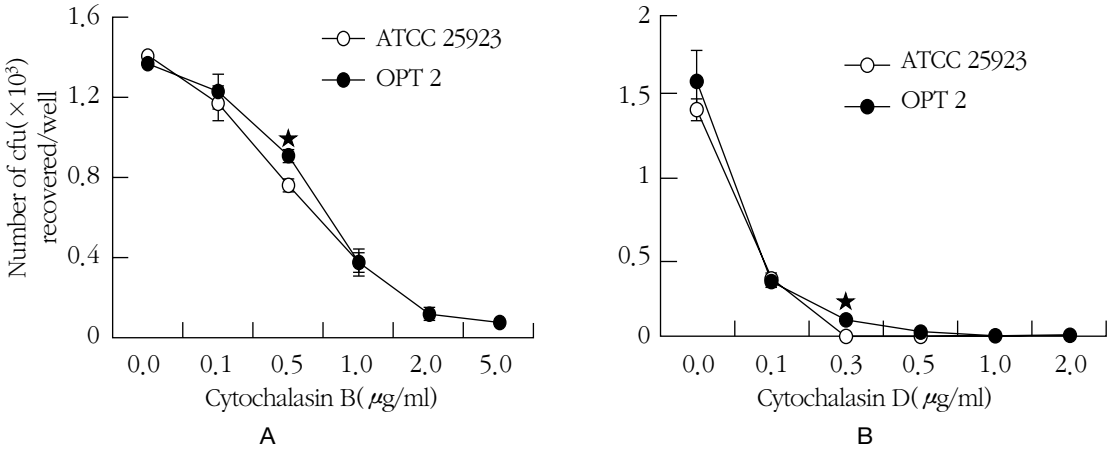

Figure 2. Dose-response of cytochalasin B (CB) (A) and cytochalasin D (CD) (B) on *S. aureus* ATCC 25923 and OPT 2 invasion to HGFs. Cell monolayers were cultured with *S. aureus* (10^5 cfu/ml) for 30 min and followed by additional co-culture in the presence of gentamicin with or without several concentrations of CB and CD for 30 min. Values represent the means±SE of 8 to 10 determinations with triplicate. The difference from the value in ATCC 25923 with CB and CD was significant at $p < 0.01$.

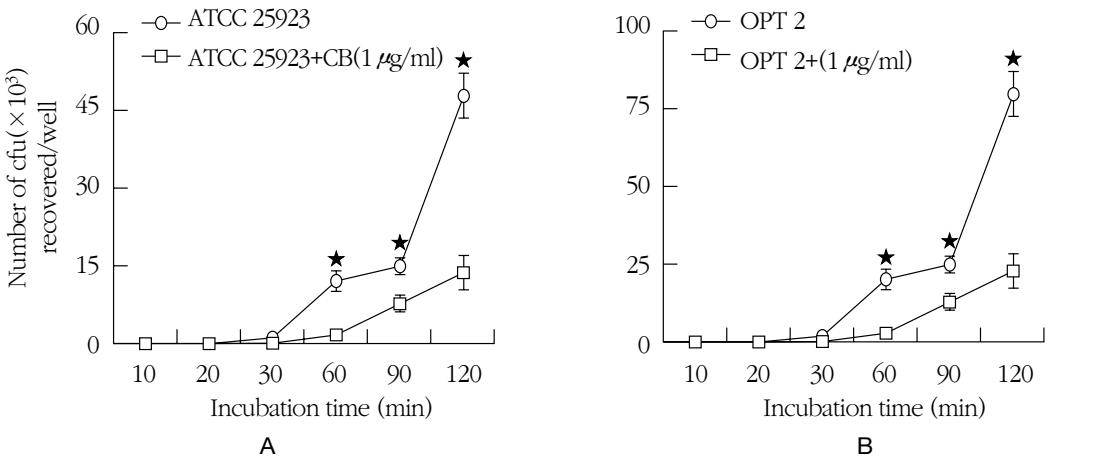

Figure 3. Inhibition of *S. aureus* ATCC 25923 (A) and OPT 2 (B) invasion to HGFs by cytochalasin B (CB). Cell Monolayers were incubated with 10^5 cfu/ml/well for several different time intervals, followed by additional co-culture with and without 1 μg/ml CB plus gentamicin for 2 hrs. Values represent the means ±SE of 8 to 10 determinations with triplicate. The difference from the value in treated cell with CB was significant at $p < 0.01$.

effects of different cell numbers and time on *S. aureus* invasion to HGFs were firstly examined by measuring the numbers of intracellular bacteria. Longer incubation time than 60 min resulted in an increase in the number of cfu recovered (Figure 1A). When HGFs were incubated for 30 min with different numbers of bacteria (1×10^2 - 1×10^7 cfu/well/ml), an increase in the inoculum size resulted in an increase in the number of cfu recovered (Figure 1B). The cfu recovered increased in a time-

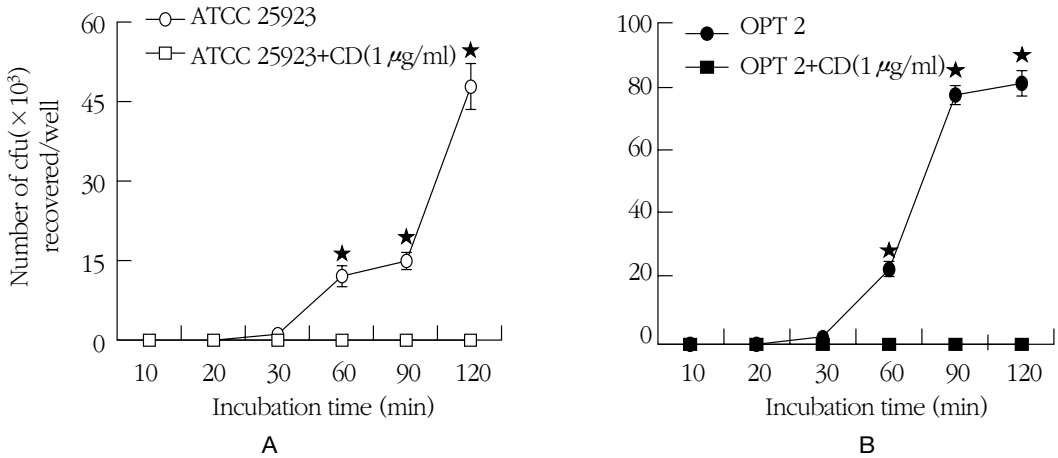

Figure 4. Inhibition of *S. aureus* ATCC 25923 (A) and OPT 2 (B) invasion to HGFs by cytochalasin D (CD). Cell Monolayers were incubated with 10^5cfu/ml/well for several different time intervals, followed by additional co-culture with and without 1 μg/ml CD plus gentamicin for 2 hrs. Values represent the means ±SE of 8 to 10 determinations with triplicate. The difference from the value in treated cell with CD was significant at $p < 0.01$.

dependent manner and approached 80% by 60 min, i.e., the percentage of the original inoculum which became bound and invaded the host cell membrane.

2. *S. aureus* invasion to HGFs

Further studies were conducted to differentiate between *S. aureus* adhesion and invasion of HGFs by using gentamicin treatment to kill adherent bacteria. Monolayers were incubated with *S. aureus* ATCC 25923 and OPT 2 (1×10^5cfu/ml) for 30min, followed by co-culture in the presence of gentamicin 10 μg/ml in the absence or presence of several different concentrations of cytochalasins (0.1-5 μg/ml)(Figure 3, 4) for 30 min. Cytochalasin decreased the numbers of *S. aureus* protected from gentamicin killing in a dose-dependent manner; 1 μg/ml CB or 0.1 μg/ml CD caused c. 50% decrease of the initially inoculated numbers of *S. aureus* (4.0×10^2cfu/ml, Figure 2). The invasion efficiency of OPT 2 was much higher than that of ATCC 25923. Additionally, cell monolayers were inoculated with 10^5cfu/well of *S. aureus* ATCC 25923 and OPT 2 for the specified times and co-cultured for 2 hrs in the presence of gentamicin with and without CB (Figure 3A,B). The recovered cfu of *S. aureus* ATCC 25923 and OPT 2 in the absence of CB increased in a time-dependent manner and approached 7.6% and 4.7% by 60 min. In comparison, CB inhibited the numbers of *S. aureus* recovered from HGFs within 30 min. Cell monolayers were inoculated with 10^5cfu/well of *S. aureus* ATCC 25923 and OPT 2 for the specified times and co-cultured for 2hrs in the presence of gentamicin with and without CD (Figure 3B). The recovered cfu of *S. aureus* ATCC 25923 and OPT 2 in the absence of CD increased in a time-dependent manner and approached 1.7% by 60min. In comparison, CD inhibited the numbers of *S. aureus* recovered from HGFs within 60 min.

3. Effect of protein A on *S. aureus* invasion

To determine the role of SPA in *S. aureus* invasion to HGFs, cell monolayers were incubated with

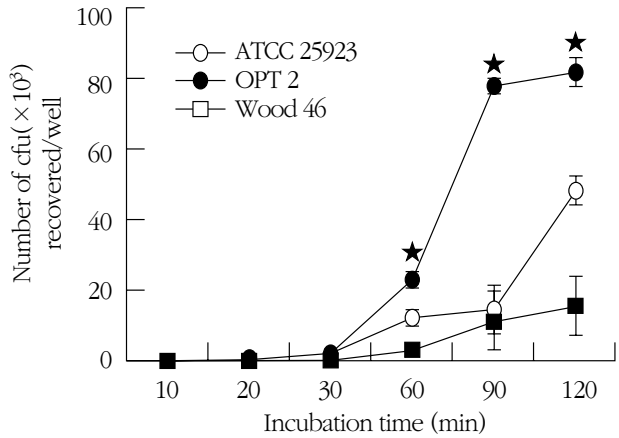

Figure 5. Efficiency of *S. aureus* ATCC 25923, OPT 2 and Wood 46 invasion to HGFs. Monolayers were infected with 10^5cfu/ml/well of each strain for specified times, followed by additional coculture for 2hrs in the presence of gentamicin. Values represent the means±SE of 8 to 10 determinations with triplicate. The difference from the value in ATCC 25923 and Wood 46 was significant at $p < 0.01$.

protein A (ATCC 25923, OPT 2) and protein A-deficient *S. aureus* Wood 46 (1×10^5cfu/ml) for the specified times, followed by co-culture with gentamicin for 2 hrs. As shown in Figure 4, the numbers of strain ATCC 25923 and OPT 2 recovered from cell monolayers increased in a time-dependent manner. However, the invasion efficiency of *S. aureus* Wood 46 remained low throughout the incubation period. At 60 min, the numbers of strain Wood 46 recovered was only 7% of those of *S. aureus* ATCC 25923 and wild type OPT 2.

The invasion efficiency of *S. aureus* to cultured HGFs was estimated by determining bacterial numbers protected from gentamicin killing for 2 hrs[24]. As shown in Figure 5, invasion efficiency increased during inoculation; 1 μg/ml CB or 1 μg/ml CD dramatically reduced its invasion efficiency. The invasion efficiency of protein A-deficient *S. aureus* Wood 46 was much lower than that of *S. aureus* ATCC 25923 and OPT 2.

IV. Discussion

S. aureus is one of the important pathogens in acute oral infection, but the pathogenic mechanism of *S. aureus* is not fully understood. In our previous study, interaction of staphylococcal protein A and cytoskeletal actin filaments was involved in the *S. aureus* invasion to cultured KB cells, and established human epidermoid carcinoma cells. To investigate the pathogenic mechanism of *S. aureus* in oral infection, the invasion of *S. aureus* in HGFs was studied. Invasion of *S. aureus* ATCC 25923 and OPT 2 was found to be time-dependent (0-120 min), and increased linearly when an increasing number of bacteria were added to media (10^2-10^7cfu/ml/well). Their invasion efficiencies were related to the structural change of cytoskeletal actin filaments. This appearance is similar to our previous studies that *S. aureus* was able to invade to oral epithelial cells[20]. This result suggests that one of the pathogenic mechanisms of *S. aureus* in oral infection may be the direct invasion of bacteria through oral epithelial cells and HGFs, which may be mediated by the interaction of staphylococcal protein A and actin filaments.

The actual mechanisms of *S. aureus* infection are provided by some authors in the host cell cytoskeleton[1, 4, 12]. The microbes make contact with the host cell plasma membrane, and this may lead to dramatic changes in the membrane at the point of contact. Eucaryotic cytoplasm has a complex internal structure consisting of protein filaments called 'microfilaments', 'intermediate filaments', and 'microtubules' that comprise the cytoskeleton. The major component of the cytoskeleton is a protein called actin. This study focuses on post-adherence events and demonstrates that internalization of *S. aureus* by HGFs utilizes microfilaments[1]. A immunoprecipitation assay with SPA-immobilized Sepharose 4B found that SPA precipitates a protein (c. 43 kDa) which has a high homology with human β-actin. This *in vitro* finding suggests that SPA has a binding affinity with cytoskeletal actin filaments of mammalian epithelial cells *in vivo*[20] and this process may be involved in the pathogenesis and invasion or internalization, or both, of *S. aureus*. Although there is no direct evidence suggesting that either mammalian actin filaments contain an SPA-binding domain, or that actin receptors are found in the surface of *S. aureus*, it is possible that the binding of SPA and actin filaments may regulate *S. aureus* infection of mammalian epithelial cells. This suggestion may be related, in part, to the previous report that intracellular pathogens develop a common mechanism to exploit the actin cytoskeleton as a means of facilitating their direct spread between cells[23].

Bacterial adhesion is typically mediated through the interaction of bacterial ligands with the surface characteristics of host cells[25-28]. For example, extracellular matrix molecules, i.e., fibronectin, vitronectin, laminin, type IV collagen and actin, can participate in the normal colonization of sites by microorganisms and in invasion during infections[29-32]. The role of cytoskeletal molecules, i.e., actin filaments, in invasion and internalization of microorganisms in mammalian cells has also been demonstrated[15,16,30,33-35]. The invasion efficiency of protein-expressing and -deficient *S. aureus* strains was significantly different. These differences suggest that unknown *de novo* processes may be involved in *S. aureus* infection in HGFs and oral epithelial cells.

It has been shown that the inhibition of actin elongation by cytochalasin concomitantly significantly inhibited the invasion of cultured HGFs by *S. aureus*[34, 36]. A similar result was also reported in renal epithelial cells[36]. However, it is possible that cytochalasin did not directly inhibit *S. aureus* invasion of cell monolayers, because the concentration of cytochalasin used in the present study had no apparent inhibitory effect on endocytosis of other microorganisms[37]. Several pahogenic intracellular bacteria, including *L. monocytogenes*, *Shigella* and *Rickettsia* spp., use host cell actin cytoskeleton for propulsion[35, 37, 38]. These suggestions indicate that protein A may be necessary for the binding of *S. aureus* to cytoskeletal actin filaments of HGFs. It is generally considered that several factors, including the infective dose, regulate bacterial invasion of mammalian cells, i.e., high levels of infecting inocula are required to elicit invasion by microorganisms that produce more chronic infections[39]. This seems to suggest that any bacterium may invade mammalian cell monolayers, if the infecting inoculum is sufficiently high. However, in the present study it was observed that the invasion efficiency of *S. aureus* Wood 46 was not increased, even when the bacterial inoculum level was increased. A similar phenomenon has been observed in *Haemophilus aprophilus* in cultured human oral epithelial cells[24]. In cultured bovine aortic endothelial cells, *S. aureus*-induced cytotoxicity depended on the size of inoculum and the length of incubation[18]. This may be due to intracellularisation of *S. aureus* and is consistent

with the results obtained here. The need for a high infective dose for invasion may indicate a different invasion tactic. The kinetics of *S. aureus* adhesion/invasion to HGFs revealed that a lag period occurred, suggesting that a factor or component involved in triggering invasion may have to attain a critical concentration for optimal invasion to occur. Furthermore, possible factors involved in *S. aureus* invasion may include bacterial and cellular metabolites or cell-surface receptors that have not been identified as yet.

In conclusion, this study has demonstrated that SPA is involved in the regulation of *S. aureus* invasion and internalization by HGFs. Elongation of actin filaments by their polymerization may mediate this process and thereby provide a novel regulatory role in *S. aureus* infection of HGFs. Similar types of *in vivo* modulation caused by a staphylococcal protein A and actin cytoskeleton may contribute to the pathogenesis of *S. aureus* infections.

V. Conclusion

These results show that *S. aureus* itself successfully invades into the internal space of HGFs and demonstrates on its invasion characteristics that *S. aureus* may invade to HGFs via cytoskeletal microfilament (actin filament).

VI. Acknowledgements

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VII. References

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-Abstract-

The purp상구균의 인체 치은 섬유모 세포에 대한 세포내 침입

김강주[1], 정규용[2]

원광대학교 치과대학 구강미생물학교실[1], 원광대학교 의과대학 약리학교실[2]

황색 포도상구균은 급성 구강 감염에 있어서의 병원균이다. 그러나 그러한 황색 포도상구균의 병원성 기전은 완전히 이해되지 않았다. 이전 실험에서 황색 포도상구균의 단백질 A와 골격근의 액틴 필라멘트는 인체 상피 세포로의 황색 포도상구균의 침입에 관여한다. 구강 내 감염에 있어서의 황색 포도상구균의 병원성 기전을 조사하기 위해 인체의 치은 섬유모 세포에 대한 침입이 연구되고 있다. 급성 구강 감염을 가진 환자로부터 분리된 ATCC 25923 황색 포도상구균과 OPT 2 황색 포도상구균의 침입은 시간(0-120 분)에 의존한다는 사실을 밝혀냈다. 60 분을 초과하는 배양시간은 다시 배양된 균집락수 증가를 가져왔다. 배지에 접종한 세균의 숫자가 증가할 때 (100 ? 10,000,000 cfu/ml/well), 직선적으로 증가한다. 단백질 A가 결핍된 Wood 46 황색 포도상구균의 침입은 단백질 A가 발현된 균주(ATCC 25923과 OPT 2)의 침입보다 훨씬 낮았다. 액틴 필라멘트의 합성을 방해하는 Cytochalasin D는 인체 치은 섬유모세포로 황색 포도상구균 (ATCC 25923과 OPT 2)이 침입하는 것을 방해한다. 이러한 결과는 구강내 감염을 일으키는 황색 포도상구균의 병원성 기전이 세포내 침입에 관여하고, 황색 포도상구균 단백질 A와 골격근의 액틴 필라멘트가 인체 치은 섬유모세포로의 황색 포도상구균의 침입 조절에 관여한다는 것을 보여준다.

주요어: 황색 포도상구균, 인체 치은 섬유모세포, 침입, cytochalasin D