Characterization of the Pathogenesis Mechanism after \textit{Pseudomonas aeruginosa} Infection through Food Consumption Using Chick Embryo Model

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Abstract

This study introduced a chick embryos’ infection model to elucidate the pathogenesis mechanism of \textit{Pseudomonas aeruginosa}, which causes serious diseases in human after ingestion of \textit{P. aeruginosa}-contaminated animal originated foods. The embryonic chick model is able to give a rapid and relatively inexpensive method to assess bacterial pathogenicity compared to embryos of other vertebrates. Embryos were infected with \textit{P. aeruginosa} and elastase-deficient \textit{P. aeruginosa}. After infection with \textit{P. aeruginosa} cells, total bacterial cell numbers and gelatinase activities in the embryos were compared. Thereafter, precartilage condensation and chondrogenesis were assessed by peanut agglutinin (PNA) binding on day 3 and by Alcian blue staining for sulfated proteoglycans on day 5, respectively. \textit{P. aeruginosa} significantly increased in embryos, resulting in abnormal limb development, whereas \textit{P. aeruginosa} defective in elastase activity partly impaired proliferation. In addition, \textit{P. aeruginosa}-infected chick embryos significantly stimulated the production of matrix metalloproteinases. Several analyses showed that elevated proteases suppressed the proliferation and survival of chondrogenic cells. The results show that this infection model was a useful assay to determine the virulence mechanism of \textit{P. aeruginosa} in human after intake of microbiologically contaminated foods.

Key words: \textit{Pseudomonas aeruginosa}, chick embryos, elastase, pathogenesis

Introduction

Bacterial infection in humans arises frequently after ingestion of contaminated foods with various pathogens, especially from pork, beef, poultry meat, and eggs (Namata et al., 2009). To escape from the prevalence of bacterial pathogens risking human health, it is necessarily preceded to undertake the problem at the level of livestock farm, thus diminishing the cross-contamination within a herd or flock (Collard et al., 2007; Namata et al., 2005). Although animals are infected with pathogenic bacteria, they become often asymptomatic, but can be spread readily at the farm (EFSA (European Food Safety Authority), 2007; Namata et al., 2009). In addition to horizontal transmissions of pathogens, bacterial infection can be vertically transmitted from mother to fetus, which is one of the important routes in the contamination of flocks with pathogenic bacteria (Namata et al., 2009).

According to previous studies, the primary genera found in pasteurized egg products are \textit{Alicaligenes}, \textit{Bacillus}, \textit{Escherichia}, \textit{Proteus}, \textit{Pseudomonas}, and Gram-positive bacteria (Schmidt-Lorenz, 1983; Cunningham, 1995). Among them, psychrotrophs including the genera \textit{Pseudomonas} primarily cause spoilage of egg whites at refrigerated condition (MacKenzie and Skerman, 1982). Thus, the shelf-life of liquid eggs is generally short at refrigeration temperatures.

Among \textit{Pseudomonas} species, \textit{Pseudomonas aeruginosa} is one of the most significant food spoilage organisms and a ubiquitous, opportunistic human pathogen which is able to cause life-threatening infections in injured, burned, and immunocompromised patients (Myszka and Czaczyk, 2009; Van Delden and Iglewski, 1998). \textit{P. aeruginosa} causes off-flavor in various foods including meats, vegetables, and fish (Bower et al., 1996). Especially, \textit{P. aeruginosa} is known to dominate proteinaceous foods including meat, poultry, milk, and fish stored at chill tem-
peratures (Gram et al., 2002). Also, ingestion of P. aeruginosa-contaminated foods may result in various diseases such as urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, and bacteremia in humans using various virulence-related factors. Among a number of virulence factors of P. aeruginosa, several determinants are required for causing disease in diverse hosts, but others only in specific host species. In addition, since P. aeruginosa is an agent of various diseases, evolutionarily divergent host infection models were developed and applied to characterize the factors and virulence mechanism of P. aeruginosa relevant to clinical settings.

Among them, genetically accessible invertebrates such as Caenorhabditis elegans (roundworm), Drosophila melanogaster (fruitfly), and Dictyostelium discoideum (amoeba) have been developed to learn P. aeruginosa pathogenesis (D’Argenio et al., 2001; Mahajan-Mihlos et al., 1999; Pukatzki et al., 2002). Unlike rodent models, these model hosts possess several advantages which include cost-effectiveness, small size and short life cycle of the organisms, thus enabling tests such as genome-wide genetic screens (Clatworthy et al., 2009). However, the use of the invertebrate hosts is unfeasible because vertebrate immune responses are distinguished from invertebrate ones, such that the latter is not related to adaptive immunity, one of the features of humans (Clatworthy et al., 2009).

A chick embryo, which is vulnerable to infection with many pathogenic bacteria, is one of critical vehicles to conduct mother-to-child transmission of bacterial infection. The embryonic chick model is capable of giving a rapid and relatively inexpensive measure of the toxicity of a number of pathogens. Chick embryos are relatively inexpensive and easy to maintain in a laboratory setting as compared with embryos of other invertebrates. Unlike other higher vertebrate systems, such as mouse ova, fertilized eggs can be sustained in a simple incubator, with need for upkeep or any manipulation of the mother; therefore expenditures of time and money for feeding and cage cleaning are unnecessary. Chicks are also ideal for developmental studies because the embryo is easily accessible and relatively easy to manipulate. Penetration of the egg and access to the developing embryo require much less time and effort than in other vertebrates. In mammals, access to the embryo cannot be acquired without manipulation of the mother, which is generally an invasive procedure that is stressful and potentially dangerous for both the mother and the developing embryo. Thus, the chicken embryo provides an excellent model for the study of the virulence of pathogens for humans, such as Neisseria gonorrhoeae and Neisseria meningitidis (Buddingh, 1970; Bumgarner and Finkelstein, 1973; Diena et al., 1975; Frasch et al., 1976). Chick embryos have been used to test host-related bacterial virulence and pathology in a wide variety of bacteria such as Francisella spp., Mycoplasma lipoferaciens, N. gonorrhoeae, N. meningitidis, Streptococcus flexneri, Escherichia coli and Vibrio cholerae (Lierz and Hafez, 2008; Nix et al., 2006; Payne and Finkelstein, 1978). The use of embryonated hens’ eggs was performed also in P. aeruginosa infection to screen antibacterial therapeutic substances as an alternative in vivo model (Hartl et al., 1997). Up to date, however, it has not been utilized to define important molecular mechanisms regarding virulence of P. aeruginosa in eggs.

Therefore, our objective in this work was to develop a useful assay system that tests the virulence and pathogenesis mechanism of P. aeruginosa, and elucidates specific features playing a major role in a human-pathogen interaction. This further will suggest an effective way to control food spoilage and vertical transmission of bacterial infection, ultimately contributing to human health.

Materials and Methods

Bacterial strains and growth conditions
Escherichia coli DH5α and P. aeruginosa PA14 strains (including wild-type, lasB and gfp mutants) were maintained on Luria-Bertani medium (LB; 10 g per liter tryptone, 5 g per liter yeast extract, 10 g per liter NaCl; Difco) at 37°C incubator. P. aeruginosa PA14 strain containing a chromosomal copy of green fluorescence protein (gfp) was created using mini-Tn7 gene integration system as previously described (Choi and Schweizer, 2006).

Bacterial inoculation into chicken embryos
Specific pathogen-free fertile eggs from White Leghorn chickens fed antibiotic-free meals were incubated in a Humidaire incubator (Saesil, Gyeonggi-do, Korea) with automatic turning, controlled temperature (37.3 to 37.8 °C), humidity (50 to 55%), and air circulation. The age of the embryos was determined morphologically by using the Hamburger series of normal stages (Hamburger and Hamilton, 1951) in the chicken embryo, with a minimum of three eggs per experiment sacrificed for this purpose.

Nine day-old chick embryos were selected for inoculation. The eggs were opened by making a window over the choioallantoic membrane and allowing the membrane to recede. They were inoculated with a sublethal infection dose, 10⁴ CFU of P. aeruginosa cells, and the window
was covered with adhesive tape. At 24 h after inoculation, suspensions of embryos, 20% by weight, were made by grinding the embryo in a Waring blender (Waring blender, Torrington, CT, USA) in cold saline buffered with phosphate. Such suspensions were centrifuged in the Swedish angle centrifuge at about 3,000 g, for 15 min before titration of the supernatant.

**Bacterial enumeration from infected embryos**

Dead embryos were removed and survived individual chick embryos were collected. The eggs were blended followed by plating dilutions of the homogenized egg extracts on LB plates to enumerate colony forming units (CFU) within infected eggs. Total bacterial numbers were standardized by dividing by each egg weight.

**Gelatin zymography**

Gelatinase activity was determined using the gelatin zymography assay with 0.1% gelatin (Sigma, St. Louis, Mo, USA) as a substrate in 10% SDS-polyacrylamide gel. Embryo lysates was subjected to SDS-PAGE under non-reducing conditions. After electrophoresis, gels were washed three times with 2.5% Triton X-100 (Sigma) in water, then incubated with 50 mM Tris buffer, pH 7.4, containing 0.2% Brij 35, 5 mM CaCl₂, and 1 mM NaCl, overnight at 37°C in a closed container. Gels were stained for 30 min with 0.25% Coomassie Blue R-250 (Amresco, Cochran Road Solon, OH, USA) in 10% acetic acid and 45% methanol, and destained for 30 min using a mixture of 20% acetic acid, 20% methanol and 17% ethanol. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

**Cell culture and cell proliferation assay**

For limb mesenchymal cell culture, the modified technique of Ahrens et al. (1993) was used. Briefly, micromass cultures used 10 µL drops of cells at 10⁷ cells/mL maintained in Ham’s F-12 medium (Gibco Invitrogen, Grand Island, NY, USA) containing 10% FBS. Micromass cultures were also incubated with or without purified gelatinase induced by inoculation. Gelatinase concentration was achieved by ultrafiltering the sample solution through Ultracel YM-30 membrane (Millipore, Concord Road, Billerica, MA, USA).

Proliferation of mesenchymal cells was determined by direct counting of cells from micromass cultures. Control and treated cultures were maintained for the indicated number of days, detached with trypsin/EDTA (Gibco, Invitrogen) solution and counted in triplicate using a hemacytometer (Superior, Lauda-Königshofen, Germany).

**Analysis of cell differentiation**

Chondrogenesis was measured by Alcian blue (Sigma) staining of sulfated cartilage glycosaminoglycans. Alcian blue-bound sulfated glycosaminoglycans were extracted with 6 M guanidine-HCl (Sigma), and quantified by measuring the absorbance of the extracts at 600 nm by spectrophotometer (Tecan, Männedorf, Switzerland).

**Results**

**Morphological defect during developmental progression of chicken embryos after infection with P. aeruginosa**

Nine day-old chick embryos were infected with various doses of wild-type P. aeruginosa and E. coli strain (EC) ranging from 10⁴ to 10⁷ CFU at an interval of a logarithmic dilution. As a result, embryonated eggs were more fatal to P. aeruginosa than to E. coli, and unlike E. coli, 10⁴ CFU of P. aeruginosa cells was shown as a sublethal dose in embryonated eggs (data not shown). At the infection dose, chick embryos were survived at 24 h after infection, while they became dead over 24 h incubation (data not shown). Therefore, chick embryos were incubated with an inoculation of 10⁴ CFU of bacterial cells for 24 h in order to observe a severe and clear effect of bacterial pathogenesis including morphological defect during developmental progression of embryonated eggs. Wild-type P. aeruginosa strain survived and proliferated fourfold more than nonpathogenic E. coli strain within chicken embryos (Fig. 1A). The bacterial infectivity was visualized with paraffin sectioning and gram staining of chicken head and body infected with P. aeruginosa cells (Fig. 1B). P. aeruginosa cells successfully established their growth within both chicken head and body.

Elastase, encoded by lasB gene, is one the most significant invasive factor during P. aeruginosa infection (Van Delden, 2004). Therefore, the virulence of wild-type and its lasB mutant were compared. Infection rates of P. aeruginosa and lasB mutant in chicken embryos were compared by recovering the embryos and enumerating bacterial cells (Fig. 1A). As a result, although wild-type and its lasB mutant grow at a same rate (data not shown), the lasB mutant displayed two-fold decreased infection rate compared to the wild-type P. aeruginosa strain. However, the infectivity of the lasB mutant was still relatively high compared to one of the E. coli strain. It is thought to be due to a relatively low level of elastolytic activity by
LasA (Kessler and Safrin, 1997).

The malformation of limb also was observed when HH stage 31 chick embryos were inoculated with *P. aeruginosa* for 24 h, suggesting the deflection on the process of endochondral ossification by infection of invasive *P. aeruginosa* (Fig. 2).

*P. aeruginosa*-induced gelatinase stimulates apoptotic death of chondrogenic progenitors

To determine whether inoculation of chick embryos with *P. aeruginosa* lead to induction of gelatinase, zymography was performed (Fig. 3A). Lysates of *P. aeruginosa*-inoculated chick embryo showed distinct bands on gelatin gels, indicating the presence of several Matrix metalloproteinase (MMP)s with gelatinolytic and/or collagenolytic activity of 30-50 kDa proteins compared to EC-inoculated embryo. This indicated that this increased activity might be responsible for virulent actions of *P. aeruginosa* including abnormal limb development. According to previous studies, corneas were destructed with infection of *P. aeruginosa* by the mechanisms that *P. aeruginosa* exproteases stimulated expression of MMPs released by corneal stromal cells and *P. aeruginosa* elastase proteolytically activated MMPs (Miyajima et al., 2001).

To confirm this, proteases activated by inoculation of *P. aeruginosa* were purified and used for determining the effects on chondrogenesis. Chondroblasts isolated from wing buds were cultured at a density of 2×10^7 cells/mL and stimulated with purified proteases. Precartilage condensation and chondrogenesis were assessed by peanut agglutinin (PNA) binding on day 3 and by Alcian blue staining for sulfated proteoglycans on day 5, respectively. With treated of *P. aeruginosa*-induced protease, PNA staining and Alcian blue uptake were significantly decreased (Fig. 3B). These results demonstrate that *P. aeruginosa*-induced protease inhibits sulfated proteoglycan accumulation and cartilage nodule formation during chondrogenesis.

Precartilage condensation is known to be regulated by cell-density due to cell proliferation or apoptotic cell death (Maini and Solursh, 1991; Solursh, 1989). We first examined if *P. aeruginosa*-induced protease-inhibited chondrogenesis was due to regulation of cell proliferation. During chondrogenic differentiation, the cell numbers increased in control cultures. Exposure of cells to *P. aeruginosa*-induced protease suppressed the proliferation of chondrogenic competent cells as determined by direct cell counting (Fig. 3C). These data indicate that the negative
regulatory action by *P. aeruginosa*-induced protease results by modulating the proliferation and survival of chondrogenic competent cells.

**Discussion**

In this study, we show that chick embryos can be utilized as an infection host for elucidating the mechanism of bacteria-mediated pathogenesis. So far, its use has been limited to test bacterial pathogenicity and to screen antibacterial drugs in various bacteria (Lierz and Haffez, 2008; Nix et al., 2006; Payne and Finkelstein, 1978; Hartl et al., 1997). However, a usefulness of its application successfully expanded into the characterization of central molecular mechanisms regarding bacterial virulence in the present study.

*P. aeruginosa* cells successfully survived and proliferated in chicken head and body for 24 h after inoculation of bacterial cells into chick embryos. The infectivity of the *lasB* mutant was relatively low compared to one of the wild-type *P. aeruginosa* strain. During *P. aeruginosa* infection, proteases play a major role in virulence and tissue penetration (Tang et al., 1996; Twinning et al., 1993). *P. aeruginosa* synthesizes several proteases, including LasA and LasB elastases, and alkaline protease. Elastin present in all invertebrates is a principal protein of connective tissues and its degradation is carried out by the LasB enzyme, which is known to be an important enzyme with elastolytic activity (Van Delden, 2004; Kessler and Safrin, 1997). Particularly, LasB elastase degrades not only elastin but also collagen, which is another major component of connective tissues (Heck et al., 1986). Elastases were determined as a main virulence determinant of causing the pulmonary hemorrhages during invasive *P. aeruginosa* clinical settings (Van Delden, 2004). Cowell et al (2003) showed that mutation of *lasB* gene encoding LasB enzyme suppressed *P. aeruginosa* invasion. In addition, previous studies demonstrated that *P. aeruginosa* lasB mutant exhibited decreased virulence in animal models (Tang et al., 1996; Tamura et al., 1992).

In addition, chick embryos suffered the morphological defect on limb during developmental progression of chicken embryos after infection with *P. aeruginosa*. It is suggested that the malformation of chicken limb resulted from the invasiveness of the bacteria by elastolytic action on embryonic connective tissues. However, how it mediates the morphological defect during limb formation is not clear at this time. At the molecular level, *P. aeruginosa* cells significantly stimulated the production of several matrix metalloproteinases, indicating the possible role in bacterial virulence such as deformed limb development. Considerably increased matrix metalloproteases played a negative role in sulfated proteoglycan accumulation and cartilage nodule formation during chondrogenesis through the down-regulation of chondrogenic cell proliferation. However, a better and complete understanding of the bacteria-mediated pathogenic mechanism should
be accomplished in future work. Taken together, chick embryo infection model was very useful to establish the bacterial virulence mechanism by examining the morphological effect and the molecular change via host-bacteria interaction. In addition, the study will provide an idea to prevent food spoilage and life-threatening disease in human by controlling bacterial infection and spread.

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References


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