

**ORIGINAL****Simvastatin represses translocation of *Pseudomonas aeruginosa* across Madin-Darby canine kidney cell monolayers**

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**Abstract :** *Pseudomonas aeruginosa* causes both invasive (bacteremic) and chronic non-invasive infections. An increase in intestinal epithelial permeability is a characteristic of severe sepsis. Alterations in the normal barrier function of the gut mucosa may result in the translocation of microbial cells and products. On the otherhand, it has been demonstrated that statin use is associated with a lower risk of mortality from bloodstream infections. Therefore, we investigated the ability of *P. aeruginosa* PAO1 to translocate across the Madin-Darby canine kidney (MDCK) cell monolayers in the presence and absence of simvastatin. The bacteria readily translocated across MDCK cell monolayers after 3 h of infection irrespective of the presence or absence of the drug in the medium. However, the bacteria were less able to penetrate the MDCK monolayers in the presence of simvastatin than in its absence. A gentamicin survival assay demonstrated that simvastatin did not affect the bacteria's invasive behavior in the MDCK cells. *J. Med. Invest.* 59 : 186-191, February, 2012

**Keywords :** *statin, bacterial translocation, MDCK, P. aeruginosa*

**INTRODUCTION**

The invasive Gram-negative pathogen *Pseudomonas aeruginosa* is a major cause of infection-related mortality among the critically ill patients (1) and represents one of the most severe nosocomial pathogens (1-4). The lungs are a major site of *P. aeruginosa* infection among critically ill patients. A significant number of such infections are caused

by direct contamination of the lungs by gastrointestinal flora or by hematogenous dissemination from the intestine to the lungs (1, 5, 6). The intestinal tract is the most important reservoir for *P. aeruginosa* in critically ill patients (7), and it has been demonstrated that a major mechanism of the lethality of intestinal *P. aeruginosa* lies in its ability to adhere to and disrupt the intestinal epithelial barrier (8).

The surprising effect of statins, a class of drugs generally prescribed for lowering cholesterol, on the incidence and severity of bacteraemic sepsis has been reported. Statins may have not only a protective effect against bacteraemic infections (9-11) but also a beneficial effect on the incidence of bacteraemic sepsis and associated mortality in critically ill patients (12) ; simvastatin inhibits *Staphylococcus*

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*aureus* host cell invasion (13). Taken together, these findings strongly suggest that statin may modulate the ability of *P. aeruginosa* to translocate across the epithelial barrier.

In this study, we explored whether or not simvastatin could modulate translocation by *P. aeruginosa* through the epithelial barrier.

## MATERIALS AND METHODS

### *Chemicals*

Dimethylsulphoxide (DMSO) was purchased from Kanto Chemical Co., Inc. (Tokyo). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chemical Company (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Wako Pure Chemical Industries Co. Ltd. (Osaka) and Equi-Tech, Inc. (Salt Lake City, UT), respectively. Triton X-100 was from Nacalai Tesque Co. Ltd. (Kyoto). Mueller-Hinton broth and Trypticase Soy Agar were from Becton Dickinson and Co. Japan (Tokyo).

### *Bacterial strain*

*P. aeruginosa* PAO1 our laboratory stock strain was used. The organisms were cultivated in Mueller-Hinton broth at 37°C.

### *Cell line and culture*

Madin-Darby canine kidney (MDCK) cells were routinely passaged in DMEM supplemented with 10% heat-inactivated FBS and 100 µg/ml kanamycin. For experimental assays, the cells were seeded at a density of approximately  $10^5$  cells/cm<sup>2</sup> in 24-well tissue culture plates or on inserts (Chemotaxicell, 6.4 mm diameter, 3 µm pore size; Kurabo Industries, Ltd., Osaka) that allow epithelial differentiation between apical and basolateral compartments. The cells were cultured at 37°C in 5% CO<sub>2</sub>-95% air atmosphere and the medium was changed daily. MDCK cells grown in 24-well tissue culture plates were incubated to early confluence (undifferentiated cells). MDCK cells grown on inserts were used at days post-confluence (fully differentiated cells).

### *Measurement of bacterial growth*

A fresh overnight culture of *P. aeruginosa* PAO1 at 37°C in MHB was diluted to  $3 \times 10^5$  cfu/ml with sterile saline. Then, 10 µl dilutions were added into 1 ml DMEM containing DMSO solution of the drug

(final concentrations : 0.1% for DMSO and 5 µM for the drug) or DMSO alone and incubated at 37°C. At intervals, 100-µl aliquots were removed and diluted appropriately with sterile saline. Dilutions (100 µl) were spread onto Trypticase Soy Agar and cultured overnight at 37°C. The colonies were then counted.

### *Cell viability by MTT assay*

Cell viability was determined by a colorimetric assay using MTT (14). In the mitochondria of living cells, yellow MTT undergoes a reductive conversion to formazan, giving a purple color. Confluent MDCK cells in 96-well plates were treated with simvastatin for 24 h. Then, the MTT solution (dissolved in phosphate buffer) was added (final concentration : 0.5 mg/ml) and the cells were further incubated at 37°C for 1 h. After the culture medium was removed, 200 µL acidified isopropanol was added to dissolve the formazan formed by the reduction of MTT. The absorbance at 570 nm was measured using a microplate reader. The percent of growth for each treatment is calculated by comparison to that determined in control cultures.

### *MDCK cell monolayer penetration assay*

The assay was performed as reported previously (15, 16). In brief, MDCK cells in DMEM with 10% FBS were seeded at  $1.5 \times 10^5$  cells per well in filter units (Chemotaxicell). Monolayers were incubated at 37°C in 5% CO<sub>2</sub> for 4 d until the transmonolayer electrical resistance (TER) reached the proper range (900-1,200 Ωcm<sup>2</sup>), as measured with a Millicell-ESR apparatus (Millipore, Billerica, MA). Monolayers were infected with bacteria by adding 10 ml ( $3 \times 10^6$  cfu) freshly grown bacteria cultured in MHB broth overnight at 37°C with shaking at 150 rpm. In some experiments, TER was monitored at several time intervals after infection to assess damage to monolayers. The assay was performed in triplicate. Each assay was repeated at least three times to confirm reproducibility.

### *Bacterial invasion assay*

With a slight modification, gentamicin survival assays were performed to quantify the bacteria invading MDCK cells (16, 17). After 1 h infection with the bacteria ( $1 \times 10^5$  cfu), monolayers were washed three times with a phosphate-buffered saline and then incubated a further 2 h in fresh medium containing 20 µg/ml of gentamicin to kill extracellular bacteria only. Monolayers were washed once with

PBS and then lysed with a 0.25% Triton X-100 for 20 min. Appropriate dilutions were spread onto agar plates, and incubated at 37°C overnight. Colony-forming units were counted to quantify bacteria surviving intracellularly.

Associated bacteria (including both adherent and invading bacteria) were measured by the addition of 1% Triton X-100 to the monolayers 3 h after infection followed by six washes without gentamicin treatment. As a control, filter units with medium only and without MDCK cells were inoculated with bacteria to determine baseline adherence to plastic. The baseline values were subtracted from those obtained by incubation with epithelial cells as described above.

#### Statistical analysis

Data were expressed as median (interquartile range) or mean  $\pm$  significant variance of the mean (SD), as indicated. The general characteristics of the two groups were tested by the Mann-Whitney U test.

## RESULTS AND DISCUSSION

The cytotoxic effect of simvastatin is shown in Figure 1. The results demonstrated that at concentrations greater than 10  $\mu$ M the drug was cytotoxic against MDCK cells in a dose-dependent manner.

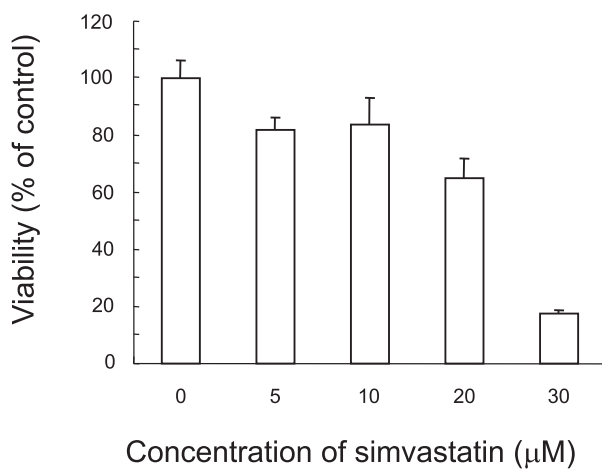


Figure 1. Effect of simvastatin on MDCK cell viability. Confluent MDCK cells in 96-well plates were treated with simvastatin for 24 h. Then, the MTT assay was performed. The absorbance at 570 nm was measured using a microplate reader. The percent of growth for each treatment is calculated by comparison with that determined in control cultures. Each value represents the mean  $\pm$  S.D. (n=4).

This means that its cytotoxicity to the MDCK cells did not appear up to a concentration of 10  $\mu$ M.

To clarify the effect of exposure of *P. aeruginosa* PAO1 to simvastatin on the growth rate, the bacteria were tested for their ability to grow in DMEM. In our preliminary experiments, the number of bacteria hardly increased in the first 3 h of incubation, and thereafter began to increase obviously. Hence, the effect of simvastatin on bacterial growth was evaluated at 1, 3 and 6 h of incubation in the presence of the drug at a concentration of 5  $\mu$ M. Compared with the result in the absence of the drug (0.1% DMSO), the two assays showed great similarities to each other in the pattern and extent of the growth of bacteria. This indicated that the drug at the concentration examined had no effect on the growth of *P. aeruginosa* PAO1 (Figure 2).

*P. aeruginosa* PAO1 translocates across MDCK monolayers (18). To confirm this, *P. aeruginosa* PAO1 was quantified in the basolateral medium at different times after apical infections of MDCK monolayers. The bacteria were not detectable after 1 h of infection. After 3 h of infection, bacteria at a population density of 4 log were detected in the absence of simvastatin (control), while the bacteria detected in the basolateral compartment remained

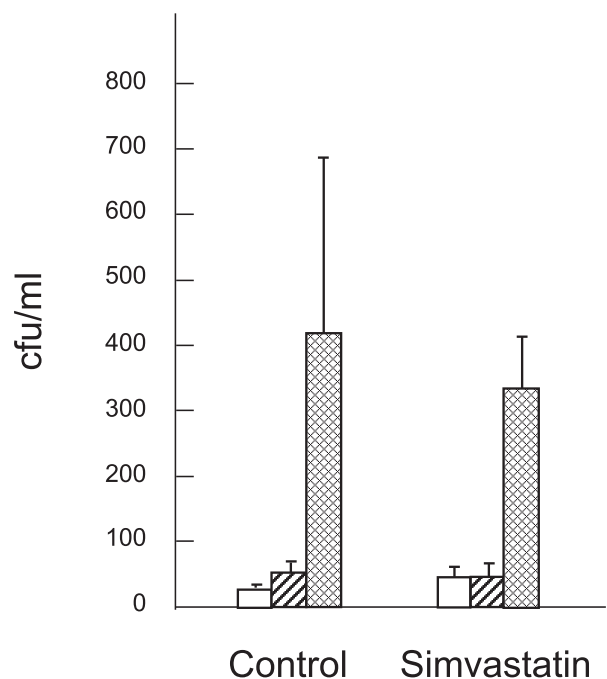


Figure 2. Effect of simvastatin on the growth of *P. aeruginosa* PAO1 in DMEM. Colony-forming units measured after 1 (open column), 3 (striped column) and 6 h (diagonally striped column) of growth in the absence (control) or presence of 5  $\mu$ M simvastatin. Data are the mean  $\pm$  S.D. (n=3).

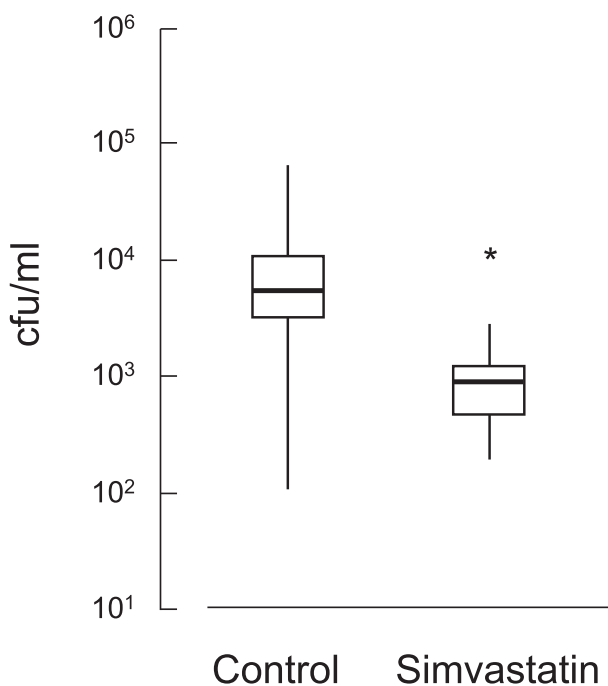
at 3 log in the presence of the drug (Figure 3). This result indicates that simvastatin repressed the bacteria's ability to translocate across MDCK monolayers.

TER is a sensitive measure of tight junctional barrier function and reflects the condition of tight junction formation. The TER values decreased as post-infection time increased. However, there was no difference in the time courses of the decrease in TER values between the presence and absence of simvastatin (data not shown). The fall in TER was independent of damages induced by acidification of the medium, because the pH of the medium was held constant during the experimental period. Based on these results, it is likely that, although *P. aeruginosa* PAO1 caused the great increase of paracellular permeability, simvastatin modulated the diminution of bacterial activity.

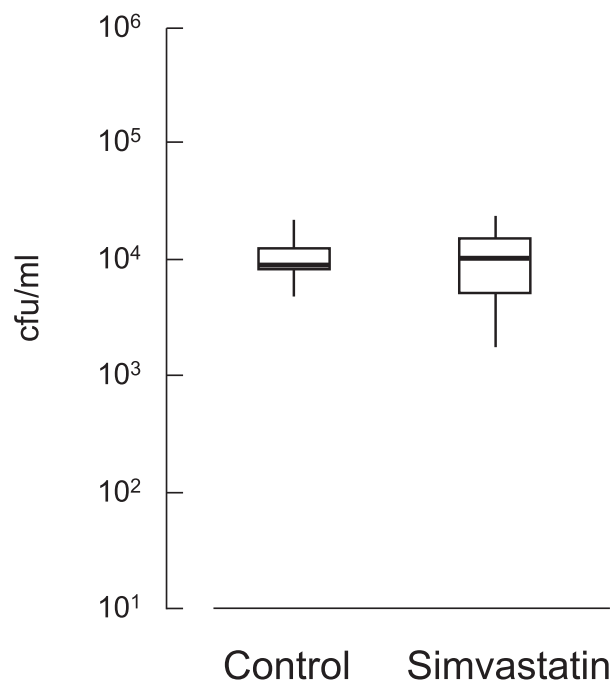
It has been demonstrated that *P. aeruginosa* PAO1 can invade epithelial cells (17), suggesting that the bacteria are able to invade MDCK cells, migrate across the cytoplasm and egress at the level of the

basal membrane. This possible transcellular translocation of *P. aeruginosa* PAO1 was investigated using a gentamicin survival assay in the presence or absence of simvastatin. It is interesting to note that, in both the presence and absence of simvastatin, *P. aeruginosa* PAO1 at a population density of 4 log was detected in the intracellular compartment of MDCK cells (Figure 4). This observation clearly demonstrates that the invasive behavior of bacteria in MDCK cells was independent of the presence or absence of simvastatin. Hence, it seems likely that simvastatin exerted certain influences to protect parts of the paracellular pathway of MDCK monolayers against damages caused by *Pseudomonas aeruginosa*, thereby repressing the translocation of bacteria, but not the flow of ions, from the apical to the basolateral compartment.

As the epithelial barrier differentiates and becomes highly polarized, it becomes increasingly resistant to *P. aeruginosa* infection (18). The integrity of the epithelial cell monolayer is maintained by junctional complexes composed of tight junctions, adherens junctions and desmosomes. The tight junctions are the most apical intercellular junctions. They form a continuous belt-like structure at the luminal



**Figure 3.** Effect of simvastatin on the ability of *P. aeruginosa* PAO1 to translocate across the MDCK cell monolayers. *P. aeruginosa* PAO1 was inoculated onto the apical surface of MDCK cell monolayers. The bacteria translocated to the basolateral compartment were quantified after 3 h of infection. Results are expressed as the median and interquartile range of the number of translocating bacteria counted after plating (cfu/ml). The two groups were compared using analysis of variance repeated measurements and least significant difference. *P*-value indicates the significance of difference between the two groups. \**P* < 0.05 is from the comparison of the two groups (Mann-Whitney U test).



**Figure 4.** Effect of simvastatin on the invasive ability of *P. aeruginosa* PAO1 in MDCK cells. MDCK cells were infected for 1 h and intracellular bacteria were quantified by plating after gentamicin had destroyed extracellular germs. Data are shown as the median and interquartile range. Analysis of variance repeated measurements and least significant difference revealed no significant difference found between the groups.

end of intercellular space that regulates the paracellular flux (19). Some bacterial pathogens can manipulate the apical-junctional complex (20).

In the present study, simvastatin reduced bacterial translocation across the MDCK monolayers in the presence of simvastatin. Unfortunately, we failed to demonstrate such an effect of simvastatin based on the results of the TER measurement and the gentamicin survival assay. A decrease in TER can result from either an increase in paracellular permeability, local cell lysis in the monolayer or a change in ion flux across the intact monolayer. It is also known that the *P. aeruginosa* quorum-sensing factor N-(3 oxodecanoyl)-L-homoserine lactone can disrupt the epithelial barrier integrity (21). Additionally, at present, there are a number of statins available. Whether the findings shown in this study are specific for simvastatin, or common with other statins, remains to be clarified. Further studies are needed to fully elucidate the role of simvastatin in the translocation process of *Pseudomonas aeruginosa* across the MDCK monolayers, and the results of these experiments may help to understand the molecular mechanism used by the bacterium to modulate the intestinal epithelial barrier function.

## CONFLICT INTEREST

None of the authors have any conflicts of interest to declare.

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