

## Stabilizing Microtubular Network Facilitates the Intracellular Growth of *Orientia tsutsugamushi*

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Received : March 20, 2006

Accepted : June 13, 2006

Microtubule network provides many intracellular microbes with an efficient way to move within host cells. *Orientia tsutsugamushi* move from the cell periphery to the microtubule organizing center (MTOC) by dynein-dependent mechanism. In this study, we investigated the role of microtubule on the growth of *O. tsutsugamushi*. The treatment of infected cells with taxol as well as daunomycin enhanced the bacterial growth in contrast to colchicine. Immunofluorescent (IF) staining of taxol-treated cells exhibited that *O. tsutsugamushi* clustered tightly near the nucleus with thick bundles of microtubules, whereas dispersed in the cytoplasm in colchicine-treated cells. These results suggest that microtubule network facilitate the growth of *O. tsutsugamushi*.

**Key Words:** *Orientia tsutsugamushi*, Microtubule, Taxol, Daunomycin, Colchicine

### INTRODUCTION

*Orientia tsutsugamushi*, a causative agent of scrub typhus, belongs to a separate genus in the family Rickettsiaceae (28), and is an endemic pathogen of many Asian-Pacific countries, including Korea (2,21). This bacterium exhibits several unique biological features, such as lack of lipopolysaccharide and peptidoglycan (1), tendency to stick to cell components (33), and the distinct ultrastructure of cell wall that differs significantly with that of its closest relatives, typhus and spotted fever group rickettsiae (24). Moreover, it leaves host cells by budding, which resembles virus (32).

Endothelial cells are one of the major target cells of *O. tsutsugamushi* infection (17). Although the cellular receptor and its bacterial ligand still remained unknown (9,21), *O. tsutsugamushi* induces phagocytosis for the invasion into cells (27). After entering host cells, it escapes the primary

phagosome into cytosol of host cells, and then moves along microtubules to the MTOC (14), where it replicates forming an aggregate near the nucleus. This movement is mediated dynein-dependent mechanism (14).

Microtubule network provides some intracellular bacteria as well as viruses with an efficient way to move inside host cells. Thus, intracellular pathogens could reach their site of replication or escape host cells to reach their new host. In addition, there are several reports that some cytotoxic chemicals with microtubule-modifying action affect the growth of intracellular microorganisms (15,20,26). Therefore, we investigated the role of microtubules on the growth of *O. tsutsugamushi*. To address this issue, we treated infected cells with microtubule-modifying agents and examined the effect of these drugs on the growth of *O. tsutsugamushi*. Our results suggest that microtubule network facilitate the growth of *O. tsutsugamushi*.

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**Abbreviations:** Microtubule organizing center, MTOC; Immunofluorescent, IF; fetal bovine serum, FBS; monoclonal antibody, MAb; fluorescein isothiocyanate, FITC.

## MATERIALS AND METHODS

### 1. *O. tsutsugamushi*

The Boryong serotype of *O. tsutsugamushi* (2) was cultivated in ECV304 cells, as described previously (12). The infected cells were cultivated until heavily infected cells detached from culture substrates. The bacterial inoculum was prepared by harvesting attached cells and subsequently disrupted with glass beads (diameter 1.0 mm). After centrifugation at 1,500 rpm for 5 min, the supernatant containing *O. tsutsugamushi* was used for the infection of ECV304 cells.

### 2. Cell culture and infection of *O. tsutsugamushi*

A subconfluent monolayer of ECV304 cells were grown on 13 mm coverslips placed into 24-well culture plates (Nunc, Denmark) at an initial cell concentration of  $1\sim2 \times 10^5$  cells per well. The cells were cultivated in M199 medium (Gibco BRL, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and incubated at 37°C in 5% CO<sub>2</sub>. This subconfluent monolayer of cells was infected with *O. tsutsugamushi* and incubated at 37°C in 5% CO<sub>2</sub>-enriched environment for 3 h, allowing sufficient time for the pathogen to establish attachment and entry into the host cells. At the end of the initial incubation period, infected cells were washed with phosphate buffered saline (PBS) and then fresh medium was added to cells.

### 3. Treatment of cells with chemicals

Subconfluent cultures of ECV304 cells were prepared before 1 day in M199 containing 10% FBS. After *O. tsutsugamushi* infection, culture medium were switched to M199 containing 2% FBS with different concentration of taxol or colchicines, and re-incubated at 37°C in 5% CO<sub>2</sub> for 3 days before the fixation for IF staining. The working concentration of each these chemicals were selected based on their effect on the cellular proliferation.

### 4. Antibodies

The methods for the production of monoclonal antibodies (MAbs) were as described previously (11). MAb FS15 is reactive against 56-kDa of *O. tsutsugamushi* (22) and MAb M686-8 is reactive against 47-kDa of *O. tsutsugamushi* (13).

For IF staining, FS15 was conjugated with fluorescein isothiocyanate (FITC) by using NHS-fluorescein (Pierce, Rockford) according to the guidelines specified by the manufacturer. MAbs against  $\alpha$ -tubulin (Clone No. DM1a, Sigma Chemical Co., St. Louis, USA) and acetylated tubulin (Sigma Chemical Co.) were used for IF staining and immunoblot experiments. Monoclonal anti- $\beta$ -actin (Sigma Chemical Co.) was used for Western blotting. For IF staining, goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Lab., West Grove, USA) conjugated to rhodamine was used with a dilution of 1:200 as a secondary antibody.

### 5. IF staining and confocal laser scanning microscopy

For the simultaneous observation of *O. tsutsugamushi* and microtubule, IF staining was performed using a MAb (FS15), which had been conjugated with FITC, and anti- $\alpha$ -tubulin. The secondary antibody was goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories) conjugated to Rhodamine. The images were taken using a confocal laser scanning system equipped with an Argon, green He-Ne, and blue laser diode mixed gas laser (Bio-Rad, Hercules, Ca.). The corresponding images were processed digitally using LaserSharp 2000 version 4.1 software (Bio-Rad).

### 6. Quantification of *O. tsutsugamushi* by counting bacterial particles

The growth of *O. tsutsugamushi* was determined as previously described with minor modifications (29). For counting particles of *O. tsutsugamushi*, ECV304 cells were infected and cultivated on a 24-well culture plate. After 3 days, infected cells were harvested and disrupted with glass beads, and then the resulting cell lysate was added to new ECV304 cells. After 3 h incubation, to allow the attachment of *O. tsutsugamushi* on the cell surface, the bacteria were stained with IF staining and photographs were taken. Quantification of the number of *O. tsutsugamushi* and cells was performed by the examination on photographs. The growth index was calculated by dividing total number of bacterial particles with total number of cells.

### 7. Western blot analysis

Cells were solubilized in lysis buffer (1% Triton X-100, 0.5% sodium-deoxycholate, 500 mM NaCl supplemented

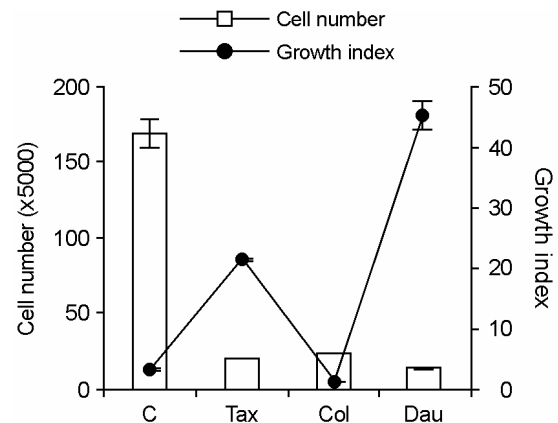
with 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, 1 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, 1 mM leupeptin). From each experiment, 25  $\mu\text{g}$  of extracted protein were separated on an 11% SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred electrophoretically to Immobilon-P polyvinylidene difluoride (PVDF)-membrane. The membranes were further incubated with primary antibodies, MAb M686-8. The membrane was incubated again with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Jackson Immuno-Research Lab. U.S.A.), and the signals were visualized using enhance chemiluminescence by Amersham ECL kit (Amersham, UK) according to the manufacturer's instructions.

## RESULTS

### 1. Effect of microtubule-modifying agents on the growth of *O. tsutsugamushi*

To address whether the microtubule structure could affect the growth of *O. tsutsugamushi*, we examined the effects of two kinds of microtubule-modifying agents: taxol and daunomycin are microtubule-stabilizing agents due to prevention of MT disassembly, and colchicine is a microtubule-destabilizing agent due to enhance of MT depolymerization. For the evaluation of the effects of these agents on bacterial growth, we first infected ECV304 cells with *O. tsutsugamushi* and then cultured for 3 days in presence of each chemicals. As the total cell number was decrease considerably by the cytotoxic action of each chemical, total growth of bacteria could not be determined. Therefore, we counted the total the bacterial particles in the surviving cells, which were affected by microtubule-modifying chemicals. The bacterial growth in the surviving taxol-treated cells was much higher than in colchicine-treated cells (Fig. 1). Daunomycin, which is known to enhance the growth of *O. tsutsugamushi* (8), also stimulated bacterial growth.

To confirm the growth-stimulating effect of taxol, we performed immunoblot analysis of 47-kDa protein of *O. tsutsugamushi* to estimate the bacterial growth. Taxol treatment resulted in the increase of 47-kDa protein in immunoblot (Fig. 2A). In contrast, colchicine treatment resulted in the decrease of 47-kDa protein in the cells treated with 5 ng/ml or higher concentrations of colchicine. Consistent with this result, the IF staining of infected cells, in which



**Figure 1.** The effect of microtubule-modifying agents on the growth of *O. tsutsugamushi*. ECV304 cells were grown to subconfluence on 24-well culture plates. After infection, cells were incubated further for 3 days in the presence of 50 nM taxol (Tax), 10 ng/ml colchicine (Col), 40 ng/ml daunomycin (Dau) and without agent (C). The number of viable cells was counted using trypan blue exclusion assay. Growth index was calculated as described in Materials and Methods. Representative result of three independent experiments is shown.

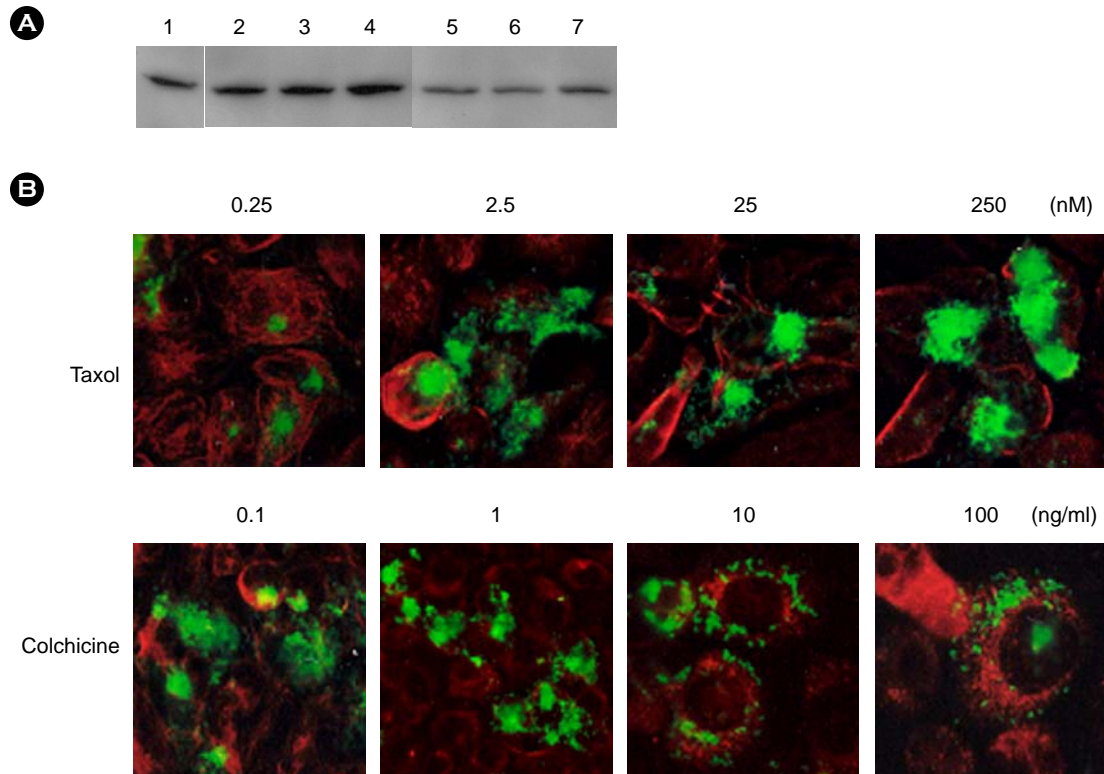
tubulin and bacteria were stained simultaneously, demonstrated that large number of *O. tsutsugamushi* clustered tightly near a nucleus along with the stiffened and thick bundles of microtubules. Furthermore this effect was seemed to be dependent on the dose of taxol (Fig. 2B). In contrast, in the colchicine-treated cells, *O. tsutsugamushi* showed a dispersed pattern in the cytoplasm and they were elongated and enlarged as compared with that in taxol-treated cells. This effect became evident in cells treated with 1 ng/ml or higher concentration of colchicine, which disintegrated microtubular network.

### 2. Analysis of acetylated tubulin

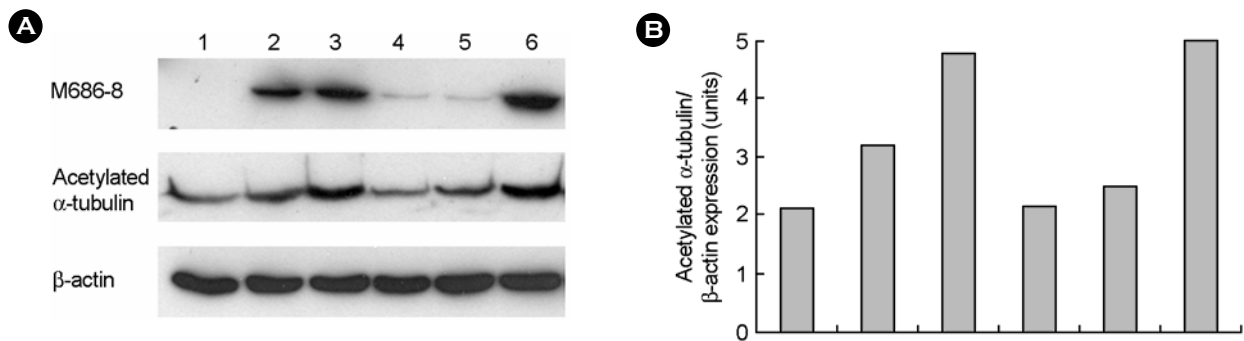
Next, we analyzed the amount of acetylated tubulin, which is known to increase the stabilized microtubules (19), and actin in cells treated with microtubule-active drugs. The data on immunoblot analysis demonstrated that treatment of cells with taxol and daunomycin increased levels of acetylated  $\alpha$ -tubulin, while the  $\beta$ -actin levels remained the same as those of the control cells (Fig. 3).

## DISCUSSION

We have observed opposite effects of taxol and colchicine on bacterial growth. Although these chemicals are cytotoxic,



**Figure 2.** The stimulating effect of taxol on the growth of *O. tsutsugamushi*. (A) The infected cells treated with taxol or colchicine for 3 days were solubilized in lysis buffer. The lysates (25  $\mu$ g) was separated on an 11% SDS-PAGE and was further analyzed with the immunoblot analysis using M686-8 for the estimation of bacterial growth. Lane 1, untreated control; lane 2, 2.5 nM taxol; lane 3, 25 nM taxol; lane 4, 100 nM taxol; lane 5, 5 ng/ml colchicine; lane 6, 10 ng/ml colchicine; lane 7, 100 ng/ml colchicine. (B) The cells treated with the indicated concentration of taxol or colchicine were stained using an anti- $\alpha$ -tubulin MAb and FS15 to stain tubulin and *O. tsutsugamushi* simultaneously. Magnification,  $\times 200$ .



**Figure 3.** Immunoblot analysis of acetylated tubulin after treatment of cells with chemicals. The infected cells treated with indicated chemicals were solubilized in lysis buffer. (A) 25  $\mu$ g of lysate was separated on 11% SDS-PAGE and was analyzed with M686-8 for bacterial protein, anti-acetylated  $\alpha$ -tubulin, and anti- $\beta$ -actin. Lane 1, uninfected ECV304 cells; lane 2, infected cells without treatment; lane 3, 50 nM taxol; lane 4, 10 ng/ml colchicine; lane 5, 5  $\mu$ M nocodazole; lane 6, 40 ng/ml daunomycin. (B) The density of each band was quantified using Quantaty One software and amount of acetylated tubulin was normalized with the density of  $\beta$ -actin.

which is evident in our experiment. However, there were changes in the microtubules as shown in IF staining of cells and the growth of *O. tsutsugamushi* was still observed in the surviving cells. Therefore, we could measure the effect

of these chemicals on the growth of *O. tsutsugamushi*. The differential effect was evident in immunoblot analysis as well as *O. tsutsugamushi* counts using IF staining. As it is well known that taxol binds to and stabilizes microtubules

by inhibiting microtubule dynamics and colchicine acts in opposite way, we hypothesized that the stabilization or destabilization of microtubules may play a specific role in the growth of *O. tsutsugamushi*. Consistent with this result, the IF staining of taxol-treated infected cells showed the large cluster of *O. tsutsugamushi* with the stiffened and thick bundles of microtubules, in contrast to colchicine-treated cells (Fig. 2B).

To confirm that microtubule stabilization enhances the growth of *O. tsutsugamushi*, we examined the acetylation of tubulin, which is known to occur on microtubules stabilized by normal cellular mechanisms (19). The data on western blot analysis has clearly demonstrated that the treatment of cells with taxol and daunomycin increased levels of acetylated  $\alpha$ -tubulin, while the levels of  $\beta$ -actin remained the same as those of control cells. Therefore, it seems evident that the treatment of taxol and daunomycin resulted in microtubule stabilization. Considering the enhanced growth of *O. tsutsugamushi* in cells treated with these chemicals, our results implicate that the growth enhancement of *O. tsutsugamushi* may be correlated with microtubule stabilization.

Daunomycin has been reported earlier to stimulate the growth of *O. tsutsugamushi* (8). However, the mechanism of action remains unknown. One possible explanation is that the growth-stimulating effect of daunomycin is due to its microtubule-stabilizing action. Daunomycin has complex mechanisms for its action. It can intercalate between base pairs of DNA, produce single- and double-stranded DNA scission, and impair DNA repair. Moreover, it has been reported to influence on the microtubule and induce its stabilization (16,18). Consistent with our explanation, daunomycin increased the levels of acetylated  $\alpha$ -tubulin (Fig. 3). Therefore, we suggest that daunomycin stimulate the growth of *O. tsutsugamushi* by its microtubule-stabilizing action.

Microtubules are the major component of cytoskeletal systems that are responsible for the regulation of distribution of cytoplasmic organelles. Microtubule-dependent clustering of mitochondria around the nucleus has been observed in various cells under experimental conditions (25). Furthermore, microtubules regulate the distribution of various organelles, namely, endoplasmic reticulum (30), Golgi apparatus (31), and lysosomes (4). They also provide tracks for

the movement of various vesicles and proteins. Considering the previous report that *O. tsutsugamushi* moves to MTOC by microtubule- and dynein-mediated movement (14), our results suggest that the proper location in cytoplasm by intact microtubules is required for the growth of *O. tsutsugamushi*.

Although host cells usually are considered to provide a suitable shelter and supply important nutrients for intracellular bacteria, cell cytoplasm may be in fact a hostile environment for intracellular parasites or bacteria (10). When, for example, extracellular bacteria are injected directly into the cytoplasm of cells, they are unable to grow. On the other hand, intracellular bacteria use specific mechanisms that enable their growth in the cytoplasm of cells (5). Thus, it seems likely that even the obligate intracellular bacterium, *O. tsutsugamushi*, requires the proper location in the cytoplasm for its survival.

It is also notable that host cell microfilaments and microtubules are known to play a critical role in the life cycles of several pathogenic intracellular microbes by providing successful invasion and promoting movement of the pathogen. In the case of *Chlamydia trachomatis* serovar L2, it was demonstrated earlier that the disruption of microfilaments as well as microtubule with colchicine or nocodazole results in the formation of multiple small inclusions instead of one large inclusion (23). In this system, microtubules are required for apposition of Golgi vesicles to chlamydial inclusions for supply of nutrients and membranes; disruption of microtubules inhibits this pathway, leading to a decrease in fusion of inclusion bodies (23). In other studies on *C. trachomatis*, bacteria require a perinuclear location for effective growth for disrupting the normal Golgi trafficking and intercepting endogenously synthesized shingomyelin, normally destined for the plasma membrane (3,6,7). Therefore, one hypothesis explaining our results is that the main purpose of location of *O. tsutsugamushi* near MTOC may be an uptake of nutrient.

In summary, we have shown that taxol and daunomycin stimulated the growth of *O. tsutsugamushi*, suggesting microtubular network stability is necessary for its intracellular life cycle.

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#### Acknowledgements

This study was supported by a grant from Inha University.

We also thank Sun-Ho Kee of the Korea University for critical review of the manuscript.

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