【原著】

Inhibitory effects of JNK on *Aedes albopictus* early larval development

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ヒトスジシマカ若齢幼虫の成長に対するJNKの阻害効果

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Abstract

The regulation of mitogen-activated protein kinase (MAPK) during the growth of early instar mosquito larvae has not been determined. In this study, we exposed early first instar *Aedes* mosquito larvae to three MAPK inhibitors (SP600125, SB203580, and PD98059). *Aedes* first instar larvae were exposed to siRNA targeted against the JNK signal transducer (JNK-siRNA). Higher larval mortality was observed after exposure to SP600125 (an inhibitor of c-Jun N-terminal protein kinase; JNK) than after treatment with the other two inhibitors. Exposure to JNK-siRNA significantly increased larval mortality at 48 h after exposure. These results suggest that the inhibition of JNK suppresses the growth of *Aedes* mosquitoes during their early larval development.

摘 要

蚊の若齢幼虫の成長におけるマイトジェン活性化プロテインキナーゼ（MAPK）の制御については明らかになっていない．本研究では，*Aedes* 属の蚊（ヤブカ）の幼虫に3種のMAPK阻害物質（SP600125, SB203580, PD98059），およびJNK（c-Jun N-terminal kinase）をターゲットにしたsiRNA（JNK-siRNA）を暴露させた．SP600125は他の2種の阻害物質に暴露させた場合よりも，幼虫の死亡率が高かった．一方，JNK-siRNAに暴露させると，幼虫の死亡率が有意に高まった．これらの結果は，JNKの阻害が，幼虫の発生初期のヤブカの成長を抑制することを示唆している．

Key words: *Aedes* mosquito (*Aedes* 属の蚊), larval development (幼虫発育), JNK

Introduction

The mitogen-activated protein kinase (MAPK) cascade is an intracellular signal transduction pathway that is evolutionally conserved and includes various members of the serine/threonine protein kinase family. In mammals, the MAPK cascade is comprised of at least four signal transduction pathways: c-Jun N-terminal protein kinase (JNK), extracellular signal-regulated protein kinase (ERK) 1/2, ERK5, and p38 (Hengartner, 2000). In particular, the

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mammalian JNK signal transduction pathway plays an important role in its development.

The whole genome sequence of *Drosophila melanogaster* was determined, and the proteins encoded by this genome were compared with those encoded by the human genome. In addition, it was indicated that the number of apoptosis-related proteins is greater in vertebrates, suggesting that they possess a more complex mechanism of development (Aravind et al., 2001). For example, the genes *jnk1*, *jnk2*, and *jnk3* have been confirmed in mammalian cells, whereas *Drosophila* possesses only one JNK protein, which is encoded by the *basket* gene (Kanda and Miura, 2004). Furthermore, this comparison of genome sequences indicated that tumor necrosis factor (TNF) and the TNF receptor (TNFR) superfamily are present in both humans and *Drosophila* (Aravind et al., 2001). Eiger, which is a TNFR found in *D. melanogaster*, modifies the morphogenesis of *Drosophila* compound eyes and wings through JNK (Moreno et al., 2002).

Mosquitoes are the most important vectors of arboviruses and malarial parasites. In view of their medical importance, mosquitoes have been the focus of considerable molecular biological research. However, there have been few studies regarding the mosquito MAPK cascade, and its precise function remains largely unknown in development.

Aedes albopictus is highly susceptible to infection with West Nile virus (WNV) (Turell et al., 2001; Sardelis et al., 2002). Indeed, WNV has been isolated from *Ae. albopictus* collected in the field (Holick et al., 2002). This suggests that *Ae. albopictus* is a vector of WNV. One of the mechanisms by which WNV is taken up into the cells of *Ae. albopictus* is endocytosis (Mizutani et al., 2003a; Chu et al., 2006), and endocytosis and phagocytosis are both regulated by JNK (Mizutani et al., 2003a). The nucleotide sequence of JNK isolated from the *Ae. albopictus* C6/36 cell line was determined (Mizutani et al., 2003b).

In common with mammalian and *Drosophila* JNK, mosquito JNK is suspected to play a key role in development. This study was examined whether inhibition of mosquito MAPK cascade, including JNK, affects the growth of early instar larvae using MAPK inhibitors and RNA interference (RNAi) techniques.

Materials and Methods

1. Mosquitoes

In this study, the *Aedes albopictus* Nagasaki colony and *Aedes aegypti* Tanzania colony were used. These two colonies were obtained from the Department of Vector Ecology & Environment, Institute of Tropical Medicine, Nagasaki University, Japan, and maintained in the insectary (controlled at 27 ± 1.0℃ and 70% ± 10% RH.) of the Microbiology Laboratory, Faculty of Pharmaceutical Sciences, Fukuoka University, Japan. The rearing density of the larvae was *ca.* 100 larvae/L, and they were kept in plastic trays (27 cm × 36 cm × 6 cm). As larval food, a 1:1 mixture of powdered mouse pellets (Clea Japan, Inc., Tokyo, Japan) and dried yeast (Tanabe Seiyaku Co., Ltd., Osaka, Japan) was supplied daily. After pupation, the pupae were collected and retained in water-filled plastic dishes (diameter, 90 mm; depth, 13 mm) in a screened cage (20 cm × 20 cm × 30 cm) until their eclosion. After all the adults had emerged, they were provided with a 3% sucrose solution as food. Mice were used for blood feeding to allow eggs to be harvested. All experiments were performed at 27 ± 1.0℃ and 70% ± 10% RH. under a 12:12 (L:D) photoperiod.

2. Bioassays

For the bioassays of the three MAPK inhibitors, early first instar *Aedes* larvae were transferred to each well of 96-well plates containing 200 μL of sterile purified water within 24 h of hatching (Nunc, Rochester, NY), and exposure experiments were performed as follows. PD98059, an inhibitor of the MEK upstream of ERK1/2; SB203580, a p38 inhibitor; and SP600125, a JNK inhibitor, were purchased from

Calbiochem Co., Ltd. (San Diego, CA). Each MAPK inhibitor was dissolved in dimethyl sulfoxide (DMSO; Wako Chemical Co., Ltd., Osaka, Japan) at a final concentration of 20 mM. The 20 mM solutions were then diluted to concentrations of 0.5 mM and 1 mM. Four mL aliquots of each dilution were added to the wells to give final exposure concentrations of 10 mM and 20 mM, respectively. In these MAPK inhibitor exposure experiments, five replicates per treatment were set up, each of which contained 24 larvae.

To examine the dose-dependent effects of exposure to SP600125, the 20 mM SP600125 solution was diluted to give seven concentrations (0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1 mM). Four mL aliquots of each dilution were added to the wells (final exposure concentrations: 4 to 20 mM). In this bioassay, five replicates—each of which contained 24 larvae—were set up for each concentration. In all MAPK inhibitor exposure experiments, 4 mL of DMSO were added as a control.

In addition, the early first instar *Ae. albopictus* larvae were exposed to small interfering RNA (siRNA) targeting JNK (Table 1). The synthesized siRNA (JNK-siRNA) was designed based on a 94–113 bp nucleotide sequence of the JNK mRNA from the *Ae. albopictus* C6/36 cell line (accession number: AF515780). Another siRNA (scrambled-siRNA), which was not homologous to any arthropod gene sequence, including those of mosquitoes, was synthesized as a control. These two siRNA were purchased from Nippon E.G.T. Co., Ltd. (Toyama, Japan). Freeze-dried siRNA was dissolved using RNase-free water in 1.5 mL sample tubes (20 mM). The siRNA solutions (56.5 mL) were dispensed into each well along with RNase-free water at a final volume of 100 mL (concentration of siRNA: 11.3 mM). Finally, a single first instar larva was transferred into each well. Ten larvae were used in each of the five replicates. RNase-free water (56.5 mL) was used as a control.

After the exposure, food was supplied daily, and the 96-well microplates were placed in a controlled incubator set at 27 ± 1.0℃ and 70% ± 10% R.H. under a 12:12 (L:D) photoperiod. The larval instars were determined every 24 h based on the presence of cast-off larval skins and size.

3. Statistical analysis

Number of death of the first instar larvae was counted and the mortality rate of the first instar larvae (mean and standard error) was calculated for each replication, after correcting the data using Abbott's formula. The significance of the difference between mortality rates was analyzed according to Zar's method (Zar, 1984). The inhibitory effect of SP600125 was determined by the IC_{50} (inhibitory concentration 50%) value, which was estimated by probit analysis.

Results

The early first instar larvae from the two *Aedes* mosquito colonies were exposed to MAPK inhibitors, and their mortality rates were determined at 144 h after their exposure (Fig. 1). When the *Ae. albopictus* larvae were exposed to 10 mM and 20 mM SP600125 their mortality

Table 1. Synthesized siRNA used for bioassay against early first instar larvae of the *Aedes albopictus* Nagasaki colony

siRNA	Sequences
JNK-siRNA	5' GCAGAAUGUCGCCAUCAAATT 3' 3' TTCGUCUUACAGCGGUAGUUU 5'
Scrambled-siRNA	5' ACUCUAGGUCGCUUAGUUGTT 3' 3' TTUGAGAUCCAGCGAAUCAAC 5'

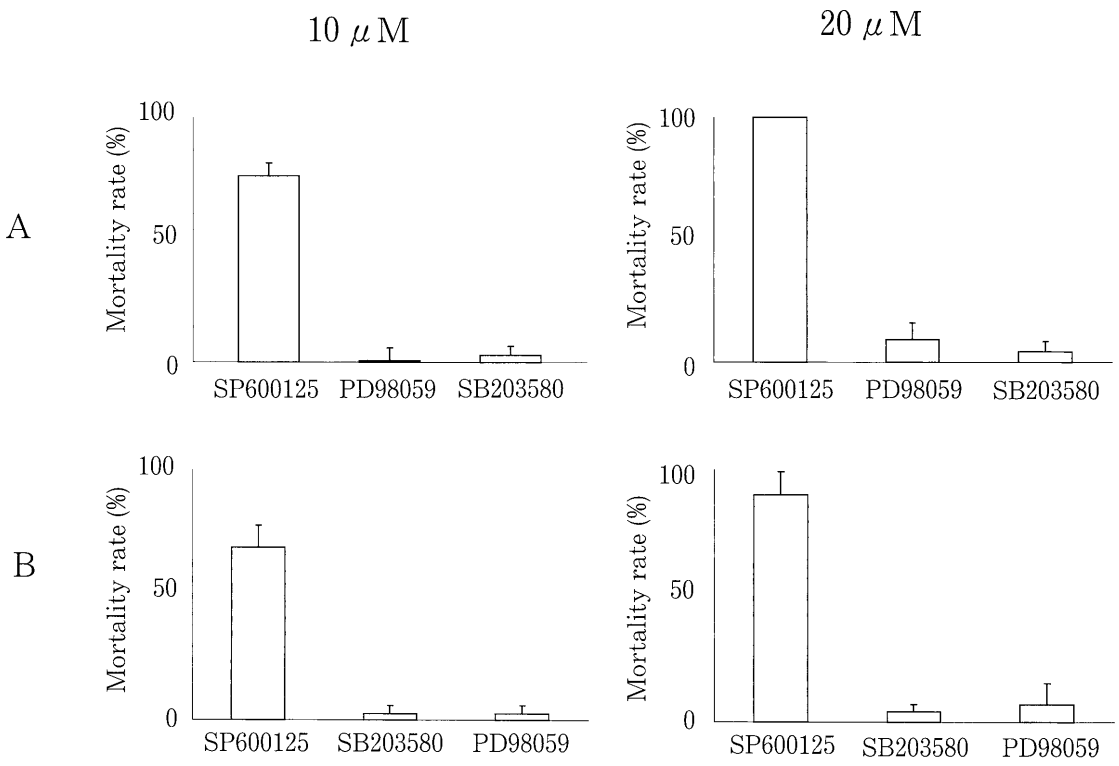

Fig. 1 Mortality rates of *Aedes* mosquito first instar larvae exposed to three MAPK inhibitors. A and B indicate the mortality rates of the first instar larvae of the *Aedes albopictus* Nagasaki colony and *Aedes aegypti* Tanzania colony, respectively.

rates were 91.6% ± 2.9% and 100% ± 0%, respectively (Fig. 1A). The mortality rates resulting from exposure to PD98059 and SB203580 were 0.9% ± 3.3% and 3.5% ± 1.3% at 10 mM and 9.4% ± 2.4% and 4.4% ± 1.6% at 20 mM, respectively (Fig. 1A). The mortality rate resulting from exposure to SP600125 was significantly higher than those associated with exposure to PD98059 or SB203580 ($P < 0.05$). There was no significant difference in the mortality rate between the larvae exposed to PD98059 and those exposed to SB203580 ($P < 0.05$).

On the other hand, when first instar *Ae. aegypti* larvae were exposed to 10 mM and 20 mM MAPK inhibitors, the mortality rates resulting from exposure to SP600125 were 69.2% ± 4.3% and 90.0% ± 4.5%, respectively (Fig. 1B). The mortality rates resulting from exposure to PD98059 and SB203580 were 2.5% ± 1.7% and 2.5% ± 1.3% at 10 mM and 4.2% ± 1.3% and 6.8% ± 4.0% at 20 mM, respectively. Similar to *Ae. albopictus* larvae, the mortality rate observed after exposure to SP600125 was significantly higher than those resulting from exposure to PD98059 or SB203580 ($P < 0.05$). The mortality rate resulting from exposure to PD98059 was not significantly different from that resulting from exposure to SB203580 ($P < 0.05$).

Subsequently, we assessed the dose dependency of the effects of SP600125 on the early first instar larvae from the two *Aedes* mosquito colonies (Table 2). As a result, we found that there were no significant differences between the regression lines of the mortality rates of the two colonies ($P < 0.05$). The IC_{50} values of SP600125 for *Ae. albopictus* and *Ae. aegypti* were 14.7 mM and 21.7 mM, respectively.

Next, early first instar *Ae. albopictus* larvae were exposed to siRNA targeting JNK, and the mortality rate of first instar larvae was calculated at 48 h (Fig. 2). In control, death larva was not observed and approximately 77% of larvae grew to second instar (Fig. 2). On the other hand, the mortality rate of the early first instar

Table 2. Regression lines and IC_{50} values for *Aedes* mosquito first instar larvae exposed to SP600125

Name of mosquito colony	Regression lines	IC_{50} (μM)
Aedes albopictus Nagasaki	Y = 2.59 log (X) + 1.98	14.7
Aedes aegypti Tanzania	Y = 1.91 log (X) + 2.44	21.7

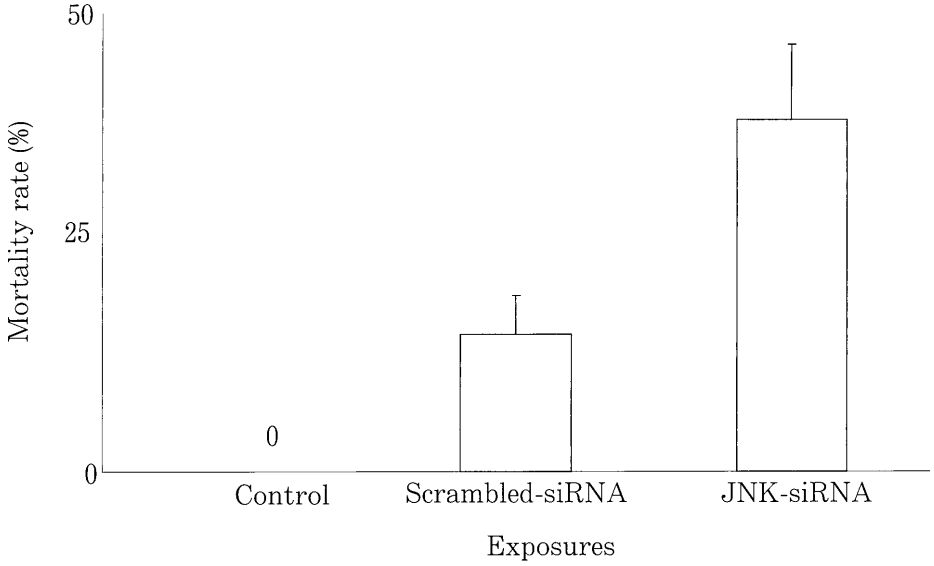

Fig. 2 Mortality rate of the first instar larvae of the *Aedes albopictus* Nagasaki colony at 48 h after exposure of synthesized siRNA-targeting JNK.

larvae increased rapidly by exposure of JNK-siRNA. In particular, difference in mortality rates resulting from exposure of JNK-siRNA and scrambled-siRNA at 48 h was significant (38.3% ± 8.3% and 15.0% ± 4.3%, respectively, $P < 0.05$).

Discussion

Among the *Aedes* mosquito larvae exposed to the three MAPK inhibitors (SP600125, PD98059, and SB203580) the highest mortality rate was detected among the early first instars exposed to SP600125. In addition, the mortality rates for both *Aedes* species exposed to SP600125 increased dose-dependently, although there was no significant difference between the IC_{50} values for the two colonies. These results suggest that mosquito JNK is one of the MAPKs that relate to growth in *Aedes* mosquito first instar larvae and that the activation of mosquito JNK is required during their early larval development. In *Drosophila*, JNK regulates in many metamorphosis events. For example, the loss of JNK activity alters the adhesion properties of larval cells and leads to the detachment of the imaginal and larval tissues during thorax closure in *Drosophila*, resulting phenotypic effects suppressing closure (Martin-Blanco et al., 2009). As well as *Drosophila* JNK, mosquito JNK might also regulate various metamorphosis events and abnormal metamorphosis, which is induced by the inactivation of JNK, might have led to the death of the first instar larvae exposed to the JNK inhibitors in the present study.

The microinjection of dsRNA, siRNA, or viral vectors has been reported to suppress the expression of target proteins in mosquitoes in a se-

quence-specific manner (Travanty et al., 2004; Boisson et al., 2006). Therefore, to confirm the role of JNK in the growth of *Aedes* mosquito first instar larvae, we exposed the larvae to siRNA targeting JNK. In this experiment, the exposure of *Aedes* larvae to the mosquito JNK siRNA resulted in a rapid elevation in their mortality rate after 48 h of exposure. Similarly, in the C6/36 cell line, a double-stranded RNA against GFP suppressed its protein expression at 48 h after transfection (Travanty et al., 2004), suggesting that exposure to siRNA targeting JNK inhibits the expression of JNK in the first instar larvae at 48 h.

This is the first report to show that, mosquito JNK, a mosquito MAPK, plays an important role in growth during early first instar larval development.

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