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▣ ORIGINAL ARTICLE

Down-Regulation of Cellulose Synthase Inhibits the Formation of Endocysts in *Acanthamoeba*

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Abstract: *Acanthamoeba* cysts are resistant to unfavorable physiological conditions and various disinfectants. *Acanthamoeba* cysts have 2 walls containing various sugar moieties, and in particular, one third of the inner wall is composed of cellulose. In this study, it has been shown that down-regulation of cellulose synthase by small interfering RNA (siRNA) significantly inhibits the formation of mature *Acanthamoeba castellanii* cysts. Calcofluor white staining and transmission electron microscopy revealed that siRNA transfected amoeba failed to form an inner wall during encystation and thus are likely to be more vulnerable. In addition, the expression of xylose isomerase, which is involved in cyst wall formation, was not altered in cellulose synthase down-regulated amoeba, indicating that cellulose synthase is a crucial factor for inner wall formation by *Acanthamoeba* during encystation.

Key words: *Acanthamoeba castellanii*, encystation, cellulose synthase, endocyst

INTRODUCTION

Pathogenic *Acanthamoeba* is a causative agent of granulomatous amoebic encephalitis and amoebic keratitis [1]. The life cycle of *Acanthamoeba* consists of 2 stages, trophozoite and cyst, and the latter is resistant to extreme physical and chemical conditions [1]. To understand the encystation mechanism and improve the treatment of amoebic infections, encystation-mediating factors have been studied. The up-regulation of 701 genes has been reported during the encystation of *Acanthamoeba* [2], and actin dynamics [3], autophagosome structure related proteins [4,5], cyst specific serine and cysteine proteases were identified [6-9]. In this study, we focused on the cyst wall related genes of *Acanthamoeba*.

The mature cyst wall of *Acanthamoeba* consists of an outer layer (the ectocyst) and an inner layer (the endocyst) separated by a space [10]. The ectocyst is composed of a combination of proteins and polysaccharides [11], whereas the endocyst is composed primarily of cellulose [12]. Therefore, cellulose synthesis is a potential therapeutic target [13]. The cellulose in *Acanthamoeba* cyst walls can also be used for a diagnosis based on CBD (the cellulose-binding domain of *Clostridium cellulovorans*) [14].

Cellulose biosynthesis in *Acanthamoeba* is achieved by converting cellular glycogen into glucose via glycogen phosphorylase [15,16]. For this purpose, glycogen phosphorylase, UDP-glucose pyrophosphorylase, and cellulose synthase are expressed at high levels during encystation [16]. Aqeel et al. [17] reported that siRNA silencing of xylose isomerase or cellulose synthase inhibits the encystation of *Acanthamoeba*, as determined by counting mature cysts resistant to SDS.

Here, we describe structural changes in the cyst wall of *Acanthamoeba castellanii* Castellani and the inhibition of encystation by transfection with siRNA against cellulose synthase (GenBank no. JX312799). In addition, we examined the effects of down-regulation of cellulose synthase on the expression patterns of xylose isomerase.

MATERIALS AND METHODS

Amoeba cultivation and encystation

A. castellanii (ATCC 30011) was obtained from the American Type Culture Collection. *Acanthamoeba* trophozoites were cultured axenically in PYG medium, which contains proteose

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peptone 0.75% (w/v), yeast extract 0.75% (w/v), and glucose 1.5% (w/v) at 25°C in a Sanyo incubator (San Diego, California, USA). *Acanthamoeba* cysts were induced in encystation media (0.1 M KCl, 0.008 M $MgSO_4$, 0.0004 M $CaCl_2$, and 0.02 M 2-amino-2-methyl-1,3-propanediol, pH 9.0) for 3 days [10]. Mature cysts were counted under a light microscope after treatment with 0.5% SDS to calculate encystation ratios [17].

Gene expression analysis based on real-time PCR

Total RNA was purified using TRIzol Reagent (Gibco BRL, Rockville, Maryland, USA), and cDNA synthesis was conducted using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Hanover, Indiana, USA). Real-time PCR was performed using the ABI PRISM® 7000 sequence detection system (Applied Biosystems, Foster City, California, USA), using the default thermocycler program for all genes [9] and sense and anti-sense primers (sense 5′-TCATCTACATGTTCTGCGCCC and antisense 5′-CGATCCAGTTGTTGAGCATGC for cellulose synthase, and sense 5′-AGTACGAGATGCTCCTCAACCG and antisense 5′-TCTCGACAA GACGAAGAGGTCC for xylose isomerase). The cDNA sequence information of xylose isomerase was obtained from differentially expressed gene analysis [7]. All reaction mixtures were made using Sybr Premix Ex Taq (Takara, Otsu, Shiga, Japan). The 18S ribosomal DNA was used as the reference gene [9]. Real-time PCR was performed to determine relative gene expressions using the 2-ΔΔCT method, and experiments were performed in triplicate.

Gene silencing

Small interfering RNA (siRNA) targeting cellulose synthase of *A. castellaniii* was synthesized by Sigma-Proligo (Boulder, Colorado, USA), based on its cDNA sequence. The siRNA duplex with sense (5′-GAUCGAGUACUUCAACAUCdTdT) and anti-sense (5′-GAUGUUGAAGUACUCGAUCdTdT) against cellulose synthase were used. The siRNA (4 μg) was transfected into *A. castellanii* trophozoites at a cell density of 4×10^5 per well as previously described [4]. As a negative control, siRNA with a scrambled sequence (Ambion, Austin, Texas, USA) absent in *Acanthamoeba* was used.

Calcofluor white staining

Acanthamoeba were incubated in encystation media for 3 days with or without siRNA against cellulose synthase. After the induction of encystation, amoeba pellets were resuspended in 2.5% calcofluor white staining solution and incubated for 20 min at room temperature [13]. After washing with PBS, samples were observed under a fluorescence microscope.

Transmission electron microscopy

Acanthamoeba were prefixed with 4% glutaraldehyde and post-fixed with 1% osmium tetroxide. Fixed cells were dehydrated with ethyl alcohol, treated with propylene oxide-resin, and embedded in resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate [9]. Samples were observed under a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).

Statistical analysis

Data are expressed as the mean ± SEM. Statistical significance was analyzed by an unpaired Student's *t*-test. A *P*-value of < 0.01 was interpreted as statistically significant.

RESULTS

Gene silencing of cellulose synthase by siRNA

Gene silencing by siRNA was used to determine the involvement of cellulose synthase (CS) in encystation of *Acanthamoeba*. The mRNA level of cellulose synthase was highly expressed at day 3 after induction of encystation (Fig. 1A-■). After transfection of CS-siRNA, a significant decrease in the mRNA expression of cellulose synthase was observed at day 3 after induction of encystation (Fig. 1A-■), showing that the encystation of *Acanthamoeba* was significantly inhibited by silencing of cellulose synthase (Fig. 1B). Scrambled negative siRNA had no effects on *Acanthamoeba* encystation.

Effect of cellulose synthase silencing on mature cyst formation

To determine the effects of gene silencing of cellulose synthase (CS) on encystation of *Acanthamoeba*, calcofluor white staining and transmission electron microscopy were performed. As shown in Fig. 2A, calcofluor staining was not observed in trophozoites. Interestingly, at day 3 post-induction, 2 different patterns of calcofluor positive materials were observed on cyst surfaces (Fig. 2B). Young cysts had a single layer of calcofluor stained, whereas mature cysts exhibited a double layer of calcofluor stained cells. An endocyst, containing a larger amount of cellulose than an ectocyst, showed a strong staining reaction (Fig. 2B, arrows). In CS-siRNA transfected amoeba, single layered young cysts predominated at day 3 post-induction, where-

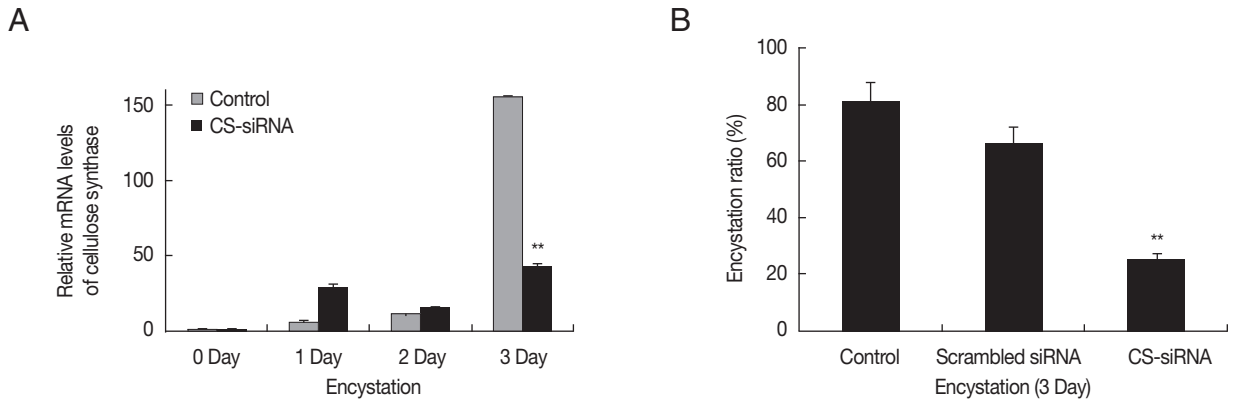

Fig. 1. Transfection of *Acanthamoeba* with siRNA against cellulose synthase. Cellulose synthase (CS) was highly expressed during encystation (A-■), but was down-regulated in CS-siRNA transfected cells (A-■). This inhibition by CS-siRNA was found to reduce encystation ratios (>50% inhibition of mature cyst formation) (B). The experiments were repeated 3 times and the average values are presented with error bars representing standard deviations. **Means were significantly different by the Student's *t*-test ($P < 0.01$).

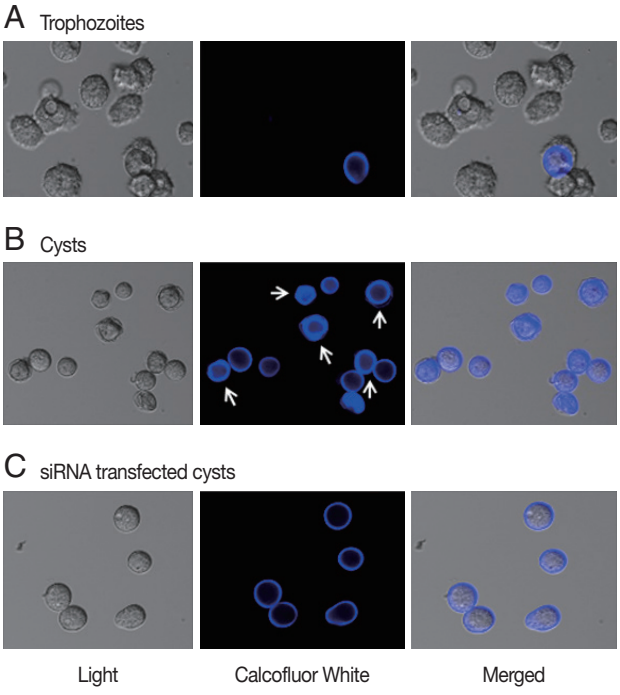

Fig. 2. Cyst wall formation as detected by calcofluor white staining. (A) Cellulose was not present in trophozoites by calcofluor white staining. (B) At day 3 after the induction of encystation, young cysts and mature cysts (arrows) were observed. (C) The majority of cellulose synthase siRNA transfected cells were young or immature cysts.

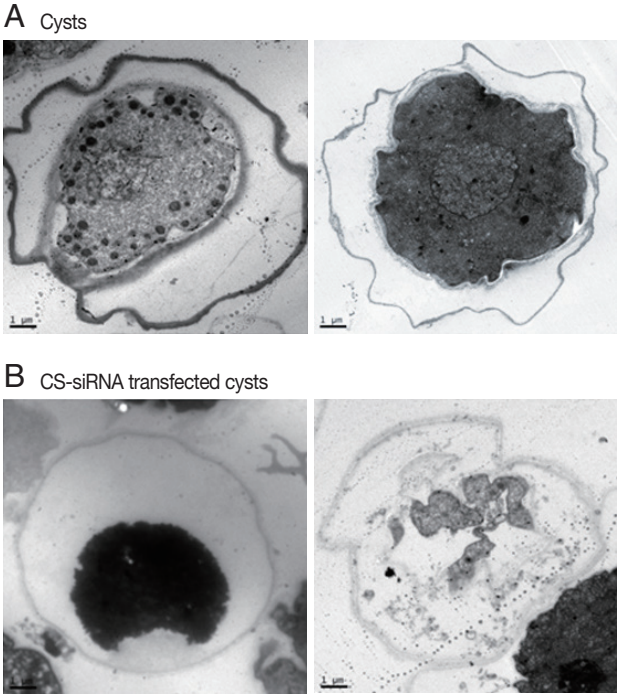

Fig. 3. Cyst wall formation as detected by scanning electron microscopy. (A) *Acanthamoeba* trophozoites were transformed into mature cysts with 2 cyst walls (the ectocyst and endocyst). (B) In contrast to normal cells, cellulose synthase siRNA transfected cells did not form 2 cyst walls and remained as young cysts or died.

as in non-transfected amoeba, double layered mature cysts were observed (Fig. 2C).

To confirm the inhibition of cyst maturation by CS-siRNA, normal mature cysts and CS-siRNA transfected cysts were observed under a transmission electron microscope (TEM). After normal induction of encystation, *Acanthamoeba* formed mature cysts with double cyst walls (Fig. 3A). Ectocysts were deeply wrinkled and electron-dense, whereas smooth-organized endocysts were closely associated with the inner cellular membrane. In contrast, CS-siRNA transfected *Acanthamoeba* showed significant impairment of cyst morphology. Cells were shrunken and lost contact with cyst walls, which lost their compactness,

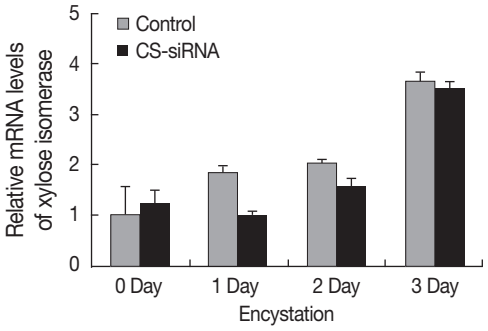

Fig. 4. The expression pattern of xylose isomerase. Xylose isomerase was expressed high in *Acanthamoeba* during encystation (▩). After transfection with cellulose synthase siRNA, the expression pattern of xylose isomerase was unchanged (■). Accordingly, the down-regulation of cellulose synthase had no effects on the expression of xylose isomerase in *Acanthamoeba*.

and ectocysts were lightly stained with few or no wrinkles. Furthermore, endocysts were not observed. The majority of CS-siRNA transfected *Acanthamoeba* remained as single walled young cysts or died (Fig. 3B).

Effects of CS-siRNA on expression patterns of xylose isomerase

Real-time PCR was conducted to examine the effects of CS-siRNA on expression of xylose isomerase in *Acanthamoeba*. Silencing of cellulose synthase during the encystation of *Acanthamoeba* had no effects on expression of xylose isomerase (Fig. 4). In fact, the expression pattern of xylose isomerase in CS-siRNA transfected amoeba was similar to that in normal untransfected controls.

DISCUSSION

During encystation, *Acanthamoeba* transforms to a double-walled mature cyst via a single-walled young cyst (immature cyst or precyst) [15], and fully matured cysts resist physical, chemical, and radiological insults [18]. For example, mature cysts are resistant to 0.5% SDS and able to excyst on agar plates, whereas single-walled precysts cannot survive this exposure and undergo lysis [15]. The resistance of mature cysts is probably due to the formation of a dense, almost impermeable wall structure [10,19].

The key component of the *Acanthamoeba* inner cyst wall is cellulose [20,21], and a efficient, rapid process for cyst wall construction involving glycogen degradation and cellulose synthesis has been hypothesized [16]. During the encystation of *Acanthamoeba*, glycogen phosphorylase and UDP-glucose pyrophosphorylase are expressed at high levels [16]. In *A. castellanii* Neff, glycogen phosphorylase knockdown caused a dramatic decrease in the number of mature cysts and increase in the number of immature cysts [15]. Furthermore, the inhibition of cellulose synthase using siRNA reduced encystation by more than 50% in *A. castellanii* Neff [17], and the cellulose synthesis inhibitor DCB (2,6-dichlorobenzonitrile) blocked the encystation of *Acanthamoeba* keratitis isolate [13]. These results suggest that targeting of cellulose synthesis could be used as a strategy for treatment of *Acanthamoeba* infection. In addition, an understanding of the role of cellulose in association with cyst wall formation would undoubtedly aid the development of novel treatments.

Our results confirmed the notion that cellulose is the main component of the inner cyst wall of *Acanthamoeba*, and showed that silencing of cellulose synthase inhibits cyst maturation. In our study, the silencing of cellulose synthase by siRNA in *A. castellanii* revealed that inner wall formation was inhibited based on the counting of mature cysts after treatment with SDS, calcofluor white staining, and electron microscopy. Furthermore, the inhibition of cellulose synthase caused young cysts to be produced and blocked progression to the mature double-walled state.

Aqeel et al. [17] proposed that xylose biosynthesis could replace cellulose biosynthesis and result in hemicellulose incorporation into the cyst wall and immature cyst formation. To confirm the relation between cellulose biosynthesis and xylose production in encystation, we analyzed the expression patterns of xylose isomerase in cellulose synthase down-regulated cells. We found that the expression level of xylose isomerase in CS-siRNA transfected amoeba was almost the same as that in normal controls (Fig. 4). Cellulose synthase and xylose isomerase are expressed independently in encysting *Acanthamoeba*, and in the present study, the expression patterns of cellulose synthase and xylose isomerase confirmed that both importantly contribute to the cyst wall formation during encystation of *Acanthamoeba*, and that one cannot replace the other.

Our observations provided an important information for those seeking to develop appropriate anti-amoebic drugs or sterilizing solutions against *Acanthamoeba*. In addition, we believe that our results could be applied to vascular plants, algae, and bacteria in which cellulose is a major cell wall component.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest related to this work.

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