Effects of short interfering RNA against methicillin-resistant 
Staphylococcus aureus coagulase in vitro and in vivo

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Objectives: The emergence of antibiotic-resistant bacteria such as Staphylococcus aureus calls for 
innovative research and development strategies. Inhibition of bacterial pathogenesis may be a promising 
therapeutic approach in this regard. The gene-silencing effect of short interfering RNA (siRNA) is useful for 
this strategy. We investigated the efficacy of siRNA on the expression of coagulase because it is the one of 
the most important enzymes in the pathogenesis of methicillin-resistant S. aureus (MRSA) infection.

Methods: We designed and synthesized 21 bp siRNA duplexes against staphylococcal coagulase. RT–PCR 
was performed to determine whether the siRNAs inhibit the expression of the coagulase mRNA and radio-
labelled siRNA was used to confirm transfection to bacteria in vitro. The efficacy of siRNA was determined 
in a murine model of haematogenous pulmonary infection.

Results: RT–PCR showed that siRNAs significantly inhibited the expression of the coagulase mRNA. The 
coagulase titres in the siRNA and control groups were 8 and 32, respectively. Measurement of incorporated 
radioactivity indicated that the siRNAs were delivered into the bacteria. In the murine infection model, in 
control and siRNA groups, 7.64 ± 0.42 and 6.29 ± 0.23 log cfu/mL (mean ± SEM) MRSA were detected, 
respectively, showing that there was a significant decrease in the number of viable bacteria in the 
siRNA group (P < 0.05).

Conclusions: The results show that siRNA inhibited both mRNA expression and the activity of MRSA 
coagulase in vitro. The in vivo results revealed that the siRNA was effective in reducing the bacterial 
load in a murine model of haematogenous pulmonary infection. Targeting of coagulase with siRNA 
appears to be a novel strategy for treating MRSA infections.

Keywords: pathogenesis, pulmonary infections, murine model

Introduction

The emergence of antibiotic-resistant bacteria and the slow progress in identifying new classes of antimicrobial agents calls for innovative research and development strategies. Current problems with resistance have arisen, in part, because nearly all antibiotics used in the clinic are derived from natural substances of microbial origin, and microbes resident in the environment possess corresponding resistance genes. These and other resistance traits have been disseminated among human pathogens following the introduction and heavy use of antibiotics. Efforts to overcome antibiotic resistance have included genetic screens to identify new drug targets and the development of antimicrobials from classes of compounds for which specific resistance traits do not exist in nature.

RNA-mediated interference (RNAi) was originally discovered as an antiviral mechanism in plants, and it was subsequently found in other organisms such as Caenorhabditis elegans, Drosophila and vertebrates.1,2 RNAi is an evolutionarily conserved process for the specific suppression of gene expression.2–4 In this process, recognition of double-stranded RNA leads to the production of small
interfering RNAs (siRNAs) of 21 to 22 nucleotides that associate with a multiprotein complex known as the RNA-induced silencing complex. This ultimately targets homologous mRNAs for degradation based on complementary base pairing. Therapeutic advantages of siRNAs are expected for the treatment of viral infection, dominant disorders, cancer and neurological disorders. However, there have not been any reports on the efficacy of siRNA against bacteria.

Methicillin-resistant Staphylococcus aureus (MRSA) infection develops mainly in inpatients with risk factors related to healthcare, although it has also been recently described in the general population. Glycopeptides, such as vancomycin and teicoplanin, are the most reliable therapeutic agents against infections caused by MRSA. However, the first report of a Japanese patient harbouring MRSA was reported in 1996.6 Fully vancomycin-resistant S. aureus first appeared in the USA in 2002.7 Community-acquired MRSA is also becoming an important public health problem.8 These reports emphasize the need for developing potent therapies other than antibiotics.

S. aureus produces many extracellular products that may act as virulence factors.9 Among these factors, staphylocoagulase has been considered to be one of the most important for virulence. We previously reported that coagulase plays a role in the development of a model of blood-borne staphylocoagulococcal pneumonia.10 Staphylocoagulase binds with prothrombin to form a complex compound called staphylothrombin that can stimulate plasma clotting by converting fibrinogen into fibrin. The formation of fibrin enhances the resistance of S. aureus against phagocytosis.10 Our data suggested that the inhibition of staphylocoagulase by siRNA may be a useful approach for controlling staphylococcal infection. In the current study, we examined the inhibitory effect of siRNA on staphylocoagulase in vitro and in vivo.

Materials and methods

Bacterial strain

S. aureus NUMR101 was isolated at Nagasaki University Hospital from blood samples of an infected patient. The bacteria were stored at –70°C in brain heart infusion (BHI) broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 10% (v/v) glycerol and 5% (w/v) skimmed milk (Yukijirushi Co., Tokyo, Japan) until use.

siRNAs

The sequences of siRNA were designed against S. aureus NUMR101 coagulase by the established methods.11 Sequences of all siRNAs tested are shown in Table 1.

All RNA oligonucleotides were synthesized by Japan Bio Service (Tokyo, Japan). The oligonucleotides were deprotected according to the manufacturer’s instructions. Equimolar amounts of complementary oligonucleotides were mixed and annealed by heating to 90°C for 1 min, followed by reducing the temperature by 2°C/min until 35°C and then by 1°C/min until the temperature reached 5°C. The resulting siRNA duplexes were analysed for completion of duplex formation by gel electrophoresis.

RNA extraction and RT–PCR

S. aureus NUMR101 was cultured on a Trypticase soy agar (BBL Microbiology Systems)-based sheep blood agar plate for 24 h at 37°C. Bacteria were suspended in endotoxin-free sterile saline and harvested by centrifugation (3000 g, 4°C, 10 min). The microorganisms were resuspended in cold sterile saline and diluted to 1 × 10⁶ cfu/mL, as estimated by turbidometry. S. aureus NUMR101 was cultured with the targeted siRNA or with a scrambled siRNA as a control. The siRNAs were added every hour. After 4 and 6 h, RNA was extracted from bacteria with a FastRNA Kit-BLUE (BIO 101, Carlsbad, CA, USA), and RT–PCR was performed to determine the level of mRNA. Oligonucleotide primers for PCR were designed according to the sequence for S. aureus NUMR101 coagulase (sense, GAC GAC ACC GAA CCC TAT TT; antisense, CAC GGA TAC CTG TAC CAG CA). PCR products were separated by electrophoresis through a 1% agarose gel containing ethidium bromide, and the signal intensity was analysed by NIH image. The 16S rRNA bands for each condition were measured, and coagulase/16S rRNA ratios were compared.

Effect of siRNA on coagulase production

S. aureus NUMR101 was cultured with targeted or scrambled siRNA. The siRNAs were added every hour. After 4 h, coagulase was assayed by a modification of the method of Jordens et al.12 Two-fold dilutions of an overnight culture in BHI broth (BBL Microbiology Systems) were prepared in sterile BHI broth to yield 100 mL volumes. To these, 0.5 mL of fresh frozen dry rabbit plasma (Eiken Chemical Co., Tokyo, Japan) diluted 20-fold with BHI broth was added. The mixtures were incubated for 2 h at 37°C. The highest dilution giving a definite clot was taken as the coagulase titre.

Measurement of the incorporation of 32P-labelled siRNA by bacteria

[32P]siRNA was prepared as described previously.13 Bacteria (1 mL; 10⁶ cfu/mL) were incubated with siRNA (30 pmol) for 4 h, after which the medium was removed and the bacteria were washed five times with PBS and centrifugation. The washed bacterial pellets were solubilized by addition of 780 μL PBS, 10 μL of 10% SDS and 10 μL of chloroform. After centrifugation (3000 g, 4°C, 10 min), the supernatant was collected as the cytoplasmic fraction. The pellet, which contained the membranes and cell walls, was resuspended in 100 μL of distilled water. The incorporated radioactivity in the various fractions was measured with a scintillation counter. For the control group, bacteria were washed immediately after treatment with radiolabelled siRNA, and cell fractions were prepared as described above.

Laboratory animals

Six-week-old pathogen-free male ddY mice (25–30 g body weight) were purchased from Shizuoka Agricultural Cooperative Association Laboratory Animals (Shizuoka, Japan). All animals were housed in a
pathogen-free environment at the Laboratory Animal Centre for Biomedical Science at Nagasaki University and received sterile food and water ad libitum. The Ethics Review Committee for Animal Experimentation at our institution approved in advance all of the experimental protocols described in this study.

Inoculum

The method of inoculation was described previously. Bacteria were pre-incubated with siRNA (2 µM) or scrambled siRNA (2 µM) for 6 h, after which they were suspended in endotoxin-free sterile saline and then harvested by centrifugation (3000 g, 4 °C, 10 min). The collected microorganisms were resuspended in cold sterile saline and diluted to 2–4 × 10^7 cfu/mL as estimated by turbidometry. The suspension was warmed to 45 °C, after which 10 mL of the suspension was mixed with 10 mL of 4% (w/v) molten Noble agar (Difco Laboratories, Detroit, MI, USA) at 45 °C. The agar–bacterium suspension (1.0 mL) was placed in a 1.0 mL syringe, and the suspension was rapidly injected using a 26-gauge needle into 49 mL of rapidly stirred ice-cooled sterile saline. This resulted in solidification of the agar droplets into beads of ~200 µm in diameter. The final concentration of agar was 0.04% (w/v), and the final number of bacteria was 2–4 × 10^7 cfu/mL. Mice were injected in the tail vein with 0.20–0.25 mL of the bacteria–agar mixture. Before the bacteria were injected, we verified their numbers by inoculating duplicates of serial dilutions onto blood agar plates and counting the cfu after incubation for 48 h at 37 °C.

Bacteriological examinations

Each group of animals was sacrificed by cervical dislocation 72 h after infection. After exsanguination, the lungs were dissected and removed under aseptic conditions. Organs used for bacteriological analyses were homogenized and cultured quantitatively by serial dilution on blood agar plates.

Statistical analysis

Bacteriological data are expressed as means ± SEM. Differences between groups were examined for statistical significance using an unpaired t-test. A P-value of <0.05 was considered statistically significant.

Results

Effect of siRNA treatment on the expression of coagulase mRNA

To determine whether siRNA can inhibit coagulase production in S. aureus, we evaluated expression of both coagulase mRNA and protein after addition of the siRNA to the bacteria. The mRNA expression (Figure 1) was dose-dependently inhibited by two different siRNAs. A high dose (2 µM) of siRNA significantly inhibited mRNA expression, and siRNA-2 was found to be more effective than siRNA-1. In addition, compared with 2 µM of the scrambled (control) siRNA, 2 µM of siRNA-2 significantly inhibited coagulase production by S. aureus (coagulase titres = 32 and 8, respectively; P < 0.05). Finally, there was no significant difference in numbers of bacteria between the siRNA and control groups (data not shown).

Incorporation of 32P-labelled siRNA by bacteria

To confirm that the siRNA were transferred to the bacteria, we measured the amount of 32P incorporated by bacteria following transfection with 32P-labelled siRNA (Figure 2). Intracellular incorporation was determined after washing and elimination of the cell wall and membrane. The radioactivity in the cytoplasm was more than 3-fold higher for the 4 h pre-treated group than the control group.

Effects of siRNA in a murine model of haematogenous pulmonary infection

We previously reported a significant correlation between the coagulase titre and the number of bacteria recovered from the lung in a murine model of haematogenous pulmonary infection.
against a lethal gene because the suppression of pathogenic factors is critical for controlling infectious diseases. Our results indicate that siRNA may present a new therapeutic option for infection by resistant bacteria via inhibition of the pathogenic process. Specifically, we showed that siRNA reduced the level of coagulase mRNA and protein in vitro and significantly lowered the number of viable MRSA in vivo. Thus, siRNA against coagulase may be able to treat MRSA lung infection. Future studies are needed to confirm this potential with other strains because only one strain was tested in this study.

However, the inhibition of mRNA expression of staphylocoagulase by siRNA was only approximately 40% compared with control. Therefore, the siRNA needs to be much more effective for clinical use. Because the [32P]siRNA uptake data revealed that a small amount of siRNA entered the bacteria, other vectors, such as liposomes, may be needed for better transfection efficacy. In this regard, lectin-facilitated liposomes, which may allow targeting of specific cell types, may be particularly useful.

We also found that the number of viable MRSA was significantly lower in the siRNA pre-treated group than the control group. We therefore examined the effect of injecting naked siRNA into the tail vein of mice 24 h after infection. However, this had no effect (data not shown). Therefore, for in vivo delivery, it may be useful to deliver the siRNA in cationic liposomes. Also, siRNA can be delivered into the lungs by rapid intravenous injection of a large volume of siRNA in PBS, but, in our case, siRNA is used to target bacteria rather than human cells. Thus, development of delivery systems to bacteria that may be compatible with human use demonstrates the potential utility of siRNA for prophylaxis and therapy of MRSA lung infection in humans. Our results indicate that siRNA may be useful for prophylaxis and therapy of MRSA lung infection in humans, but application will require the development of bacteria-specific delivery systems that are compatible with human use.

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Transparency declarations

None to declare.

References


