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Original article

MicroRNA fragments derived from *Streptococcus pyogenes* enable activation of neutrophil phagocytosis: *in vitro* study

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Abstract

MicroRNAs are single-stranded RNAs that regulate gene expression by forming imperfect base pairs, which have also been speculated to play regulatory roles in gene expression of *Streptococcus pyogenes* itself. We hypothesized that bacterial microRNAs cause molecular interference in host, when there is high homology to human microRNAs. Total RNA from cultured *S. pyogenes* strain SSI-1 was isolated and the cDNA fragments were then inserted into vector plasmid and transformed to competent cells, after which genomic sequence analyses were performed. Cell transfection, evaluation of mRNA transcription, measurement of inflammatory mediators, and assessment of surviving bacteria with murine splenocytes were also performed. Three microRNAs were selected from about 600 candidates according to their homology with human genome DNA. In the quantitative method, transcription of nasopharyngeal cells with microRNA was significantly lower in 2 of 11 targets, and greater in 10 of 11 targets. The ELISA findings revealed that transcription of MIP-2 was significantly greater with miR-SSI1-221 and miR-SSI1-281. Furthermore, strain SSI-1 had significantly higher survival in the supernatant of the control as compared to the miR-SSI1-221 and miR-SSI1-281 transfected cells. In conclusion, microRNA fragments derived from *S. pyogenes* have a high homology to the human genome and contribute to enhancement of the host immune system.

Keywords: *Streptococcus pyogenes*; MicroRNA; MIP-2; Neutrophil

1. Introduction

Streptococcus pyogenes (group A Streptococcus) is an important human pathogen that causes a variety of clinical manifestations ranging from noninvasive diseases, such as pharyngitis and impetigo, to more severe invasive infections, including necrotizing fasciitis, sepsis, and streptococcal toxic shock syndrome [1]. Streptococcal pharyngitis is commonly seen in children and adolescents, and most cases are caused by *S. pyogenes*. A number of antibiotics have been shown to be effective in treating streptococcal pharyngitis, including penicillin and its congeners (such as ampicillin and amoxicillin), as well as numerous cephalosporins, macrolides, and clindamycin. Nevertheless, penicillin remains the treatment of choice, because of its proven efficacy and safety, narrow spectrum, and low cost [2,3]. However, antibiotic treatment failure in clinical cases of streptococcal pharyngitis has been reported [4,5]. In addition, several recent studies have warned that macrolide resistant *S. pyogenes* strains are increasing worldwide [6–8]. Thus, establishment of a new treatment method different than current therapy that is mainly based on antibiotics is urgently required.

MicroRNAs are single-stranded RNAs of 22 or fewer nucleotides in length that post-transcriptionally regulate gene

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expression by forming imperfect base pairs. They function as guide molecules in post-transcriptional gene regulation by base-pairing with target messenger RNAs, usually in the 3′ untranslated region [9,10]. More than 1000 microRNAs have been identified in mammals and found to be associated with diverse biological processes, such as cell differentiation, metabolism, tumorigenesis, and immunity [11–13]. To date, microRNAs are thought to regulate gene transcription and translation. Most microRNAs produced by *S. pyogenes*, which has an ability to invade human epithelial cells, have also been speculated to play regulatory roles in gene expression of *S. pyogenes* itself [9,14,15]. In addition, Perez et al. showed that abundant small RNAs are transcribed throughout the *S. pyogenes* genome [16]. With that in mind, we hypothesized that bacterial microRNAs cause molecular interference in host microRNAs when there is high homology between the molecules, since some nucleotide fragments from invaded bacteria including *S. pyogenes* may remain in the host cells and the nucleotides might contain microRNAs. However, only limited data are available to describe the effects of *S. pyogenes* on the expression of microRNAs in inflammatory sites. MicroRNAs from *S. pyogenes* with high homology to human microRNAs may enhance the immune system of the human host. In this study, we aimed to identify microRNAs that promote host immune activities and investigated whether those molecules can contribute to the control of infectious diseases.

2. Materials and methods

2.1. Cell invasion assay

Human nasopharyngeal epithelial Detroit 562 cells (isolated from a human pharynx carcinoma, Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) were cultured in flasks or 24-well plates containing Minimum Essential Medium alpha (MEMα; Sigma–Aldrich Inc., St. Louis, MO), supplemented with 10% fetal bovine serum (FBS; Life Technologies Corp., Carlsbad, CA) and 20 μg/ml of gentamicin. Detroit 562 cells were incubated at 37 °C in a 5% CO_2 atmosphere. Internalization of *S. pyogenes* into Detroit 562 cells was assessed using an antibiotics protection assay [17]. Briefly, cultures were grown in THY medium and harvested at the mid-exponential phase of growth ($A_{550} = 0.30–0.60$) by centrifugation for 5 min at 5000× *g*, then washed once and resuspended in MEMα supplemented with 10% FBS. Detroit 562 cells were infected with *S. pyogenes* strains at a multiplicity of infection (MOI) of 10 for 2 h. Bacterial density was confirmed by plate counts. For assessment of bacterial internalization, the cells were washed with MEMα, then incubated with MEMα supplemented with gentamicin (100 μg/ml) and penicillin G (100 U/ml) for 1 additional hour. The cells were then washed and burst with sterilized water, and bacterial numbers were counted following inoculation onto THY agar plates.

2.2. Isolation of S. pyogenes microRNA

Total RNA from cultured *S. pyogenes* SSI-1 (*emm3*) was isolated using a Maxwell 16 Total RNA Purification Kit (Promega Corp., Madison, WI) according to the manufacturer's protocol. Total RNA was immediately synthesized to complementary DNA (cDNA) using a SuperScript III First-Strand Synthesis System SuperMix (Life Technologies Corp.). The cDNA fragments were then inserted into pGEM-T Easy Vectors (Promega Corp.) and transformed to XL-10 Gold Ultracompetent Cells (Agilent Technologies Inc., Santa Clara, CA), after which genomic sequence analyses were performed. Fragments with a high homology to human microRNA were purified using a Miniprep Kit (Life Technologies Corp.). After cleavage with *Bam*HI or *Hin*dIII, each fragment was inserted into pBApo-CMV Neo DNA (Takara Bio Inc.).

2.3. Cell transfection

All transfection procedures in this study were performed using Lipofectamine 2000 Transfection Reagent (Life Technologies Corp.) according to the manufacturer's instructions. Briefly, 2×10^5 Detroit 562 cells were plated in 100 μl of serum per well without antibiotics. In each well, 1 μg of plasmid DNA was added after dilution with 50 μl of OptiMEM I Medium (Life Technologies Corp.) and 2 μl of Lipofectamine, then gently mixed and incubated for 5 min at room temperature. Next, plasmid–lipofectamine complexes were added directly to each well containing Detroit 562 cells or murine splenocytes, and incubated at 37 °C in a 5% CO_2 atmosphere. Finally, the cells were selected by adding 500 μg/ml of neomycin to the medium.

2.4. Evaluation of mRNA transcription using real-time PCR

Real-time PCR and data analyses were performed using a total volume of 20 μl in 96-well reaction plates with a StepOnePlus Real-Time PCR System (Life Technologies Corp.). Each reaction tube contained 10 μl of Fast SYBR Green Master Mix (Life Technologies Corp.), a 1-μM primer set for the target genes, and 10 ng of sample DNA in a 20-μl PCR mixture. The primers used for real-time PCR analyses are listed in Table 1.

2.5. Measurement of inflammatory mediators

Concentrations of inflammatory mediators were quantified in cell-free supernatants of cultures using a Quantikine Mouse Cytokine Detection System (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Each sample was assayed in triplicate, and cytokine standards and quality controls supplied by the manufacturer were run on each plate. The multiplex assay was performed twice using cell-free culture supernatants from different experiments. Data were acquired for optical density at 450 nm.

2.6. Assessment of surviving bacteria with murine splenocytes

MicroRNA samples were transfected to murine splenocytes in 1 well of a μ-Slide (NIPPON Genetics Co. Ltd., Tokyo, Japan)