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Original article

Extraordinary GU-rich single-strand RNA identified from SARS coronavirus contributes an excessive innate immune response

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Abstract

A dangerous cytokine storm occurs in the SARS involving in immune disorder, but many aspects of the pathogenetic mechanism remain obscure since its outbreak. To deeply reveal the interaction of host and SARS-CoV, based on the basic structural feature of pathogen-associated molecular pattern, we created a new bioinformatics method for searching potential pathogenic molecules and identified a set of SARS-CoV specific GU-rich ssRNA fragments with a high-density distribution in the genome. *In vitro* experiments, the result showed the representative SARS-CoV ssRNAs had powerful immunostimulatory activities to induce considerable level of pro-inflammatory cytokine TNF-a, IL-6 and IL-12 release via the TLR7 and TLR8, almost 2-fold higher than the strong stimulatory ssRNA40 that was found previously from other virus. Moreover, SARS-CoV ssRNA was able to cause acute lung injury in mice with a high mortality rate *in vivo* experiment. It suggests that SARS-CoV specific GU-rich ssRNA plays a very important role in the cytokine storm associated with a dysregulation of the innate immunity. This study not only presents new evidence about the immunopathologic damage caused by overactive inflammation during the SARS-CoV infection, but also provides a useful clue for a new therapeutic strategy.

Keywords: SARS; Viral pathogenesis; ssRNA; TLR7; TLR8; Inflammation

1. Introduction

SARS coronavirus (CoV) is a novel etiological agent resulting in an atypical pneumonia followed with severe acute respiratory syndrome (SARS). With the whole genome sequencing of SARS CoV, the proteins (S, E, M, and N) have been identified [1,2], and their structure and function associated with evasion, virulence and immunity have been studied in great detail [3–6]. Although much has been learned since its outbreak in 2003, many aspects of the pathogenesis of the disease remain obscure [7].

Viral interactions with the host immune system always play a central role for the outcome of infection. Because cytokine storm has been observed in the rapid course of SARS [8], and the pro-inflammatory cytokines, such as IL-6, TNF-a, etc, are up-regulated in clinical serum or cultured supernatants [9–13], it is inferred that an overactive innate immune response should contribute to virus-induced immune pathology resulting in acute lung injury in SARS patients. Therefore, more detailed knowledge about how the viruses interact with the host innate immune system is very important for understanding the molecular mechanisms of pathogenesis and progress of the disease [14].

During the virus invasion, host toll-like receptors (TLRs) are able to recognize different pathogen-associated molecular patterns (PAMPs) and trigger innate immune response [15]. Recently, the spike (S) protein has been proposed to be recognized by TLR2 and provoke the pro-inflammatory cytokines release [16–18]. However, little is known about the role of single-strand RNA (ssRNA) in the SARS-CoV as a kind of potential PAMP. Since a GU (guanosine and uridine)

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rich ssRNA40 from the U5 region of HIV-1 was identified as a natural agonist of TLR7 and TLR8 [19], it is attractive if there are immune stimulatory ssRNAs existed in about 30 kb length of single-stranded SARS-CoV RNA genome, and the relevant immune effect is still unrevealed.

In this study, we attempt to make a comprehensive investigation on the SARS GU-rich ssRNA in the SARS-CoV RNA genome by bioinformatics scanning technique, evaluate their effect in the host innate immune response in order to reveal more detailed immunopathogenetic mechanism.

2. Materials and methods

2.1. TLR agonists

The phosphothioate-protected ssRNAs were synthesized by IDT, inc. (IA, USA), including ssRNA40 (5′-GCCCGUCUGUUGUGUGACUC-3′; at U5 region 108–127 nt of HIV-1 genome), ssRNA120 (5′-GUCUGAGUGUGUUCUUG-3′; at 24,524–24,540 nt in the SARS-CoV genome), ssRNA83 (5′-GUG CUUGUGUAUUGUGC-3′; at 16,174–16,190 nt in the SARS-CoV genome), and the U/A alternated fakeRNA120 (5′-GACAGAGAGAGAACAAG-3′). DOTAP (Liposomal Transfection Reagent *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate, Roche, Inc.), a liposomal transfection reagent, was used to complex with ssRNA before addition to cell cultures. The DOTAP/ssRNA mixture was prepared according to the following procedure: the ssRNA was diluted by HBS (HEPS-buffered saline) to a concentration of 0.025–0.6 μg/μl and the DOTAP was dilute by HBS with the ratio of 1:3, then the diluted ssRNA and DOTAP were mixed at the ratio of 1:2. In addition, Pam3CK4 were purchased from Invivogen (San Diego, USA) as a TLR2 agonist.

2.2. Cell culture and stimulation with TLR agonists

The mouse macrophage-like cell line, RAW264.7 (American Type Culture Collection), was fed in DMEM medium (GIBCO, USA), the human acute monocytic leukemia cell line, THP1 (American Type Culture Collection), was fed in RPMI1640 (GIBCO, USA), the human PBMCs were isolated from normal human peripheral blood by a density gradient centrifugation with Lympholyte-H medium (Cedarlane Laboratories, Ltd., Canada), and resuspended in RPMI 1640. In addition, the culture media supplemented with 10% (v/v) low-endotoxin fetal calf serum (FCS) (Hyclone, USA), and 100 IU/ml penicillin and 100 g/ml streptomycin as well at 37 °C in a 5% CO_2 humidified incubator. Live cells diluted in the PBS (0.1 mM, pH 7.2) with 0.4% trypan blue were counted by a hemocytometer.

The prepared cells were resuspended at 1×10^6/ml in 0.2 ml culture media in 96-well plates. Before the addition of TLRs agonists, 4 h culture was needed for the adherence of RAW264.7 cells but not for THP1 cells. Each group consisted of four-well repeats for each sample. The group treated with ssRNA40 at a final concentration of 5 μg/ml was denoted as positive control of TLR7/8 stimulation, and that treated with 5 μg/ml fakeRNAs as the negative controls. Each of the experiment groups was treated respectively with 5 μg/ml different GU-rich ssRNA for an independent stimulation. A comparative analysis of immunostimulatory activity was performed between ssRNA40 and ssRNA120 respectively at the concentration gradient of 0, 1.25, 2.5, 5, 10 and 20 μg/ml.

2.3. Cytokine ELISA for TNF-a, IL-12, IL-6 and IFN-a

The above groups were cultured at 37 °C in a 5% CO_2 humidified incubator, the supernatants at each well were collected after 10 h culture for the measurement of TNF-a level, and collected once again after 24 h culture for the measurement of IL-6, IL-12 and IFN-a levels.

The cytokines were detected by the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocols of the relevant ELISA kits, The ELISA kits used in this study included the anti-mouse or anti-human TNF-a and IL-6 ELISA kit (eBioscience, Inc., USA), IL-12p40 ELISA kit (Boster, Inc., China) and IFN-a ELISA kits (Mabtech inc., USA). The cytokines were measured at 450 nm wavelength by Varioskan Flash spectral scanning multimode reader (Thermo Scientific, USA). Finally, one of representative experiment of at least two independent experiments would be shown in the results.

2.4. Gene knockdown

To knockdown the TLR7 and TLR8, the siRNAs templates were designed by BLOCK-iT™ RNAi Designer (Invitrogen inc.) based on the TLR7 and TLR8 mRNA sequences (GenBank accession no. NM_133211.3 and AF246971.1) and synthesized in Invitrogen inc. A preliminary test selected the best one for gene knockdown out of the three candidate templates from TLR7 (NM_133211.3_stealth_1560 5′-AAUAGUGUAAGGCCUCAAGGACCUG) and that from TLR8 (AF246971.1_stealth_1520 5′-AAGAGGAACUAUUUGCAUAACUCUG).

According to the standard siRNA transfection protocol provided by Invitrogen inc, the siRNA (5 pmol/ml) was transfected into cells in 96 wells plate (the cells are 60–80% confluent for each well) by Lipofectamine™ 2000 for 24 h culture, Then each well was replaced with fresh 1x normal growth medium, Meanwhile, the experiment groups were respectively treated with 5 μg/ml ssRNA120 and ssRNA83. The TNF-a levels were detected by ELISA after another 24 h culture.

2.5. Animal model and histological study

Adult male Kunming mice (18 ± 0.5 g) were obtained from the Animal Center of the Third Military Medical University (Chongqing, China) under the approval by the Animal Care and Use Committee. All the mice were injected with 800 mg/kg of D-GalN via tail vein [20]. One hour later, the mice were randomly divided into two groups ($n = 10$ for each), the mice in control group took a tail vein injection of 300 μl HBS and