



The modulation of Th2 immune pathway in the immunosuppressive effect of human umbilical cord mesenchymal stem cells in a murine asthmatic model

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Abstract

Background Asthma is a chronic airway inflammatory disease that has a high prevalence nowadays, and seeking the means of relieving asthmatic symptoms is now an issue with increased importance. While mesenchymal stem cells have been demonstrated to display immunomodulatory effects, the effect of fetus-type mesenchymal stem cells (MSCs) on asthmatic symptoms in vivo have not been reported to date.

Methods Female BALB/c mice at 8 weeks of age were sensitized by ovalbumin, and MSCs derived from Wharton's jelly of human umbilical cord mesenchymal stem cells (hUCMSCs) were injected into the asthmatic mice. Airway hyper-responsiveness, lung eosinophil infiltration, cytokine level in splenocyte cultures and serum

immunoglobulin level were measured. Enzyme-linked immunosorbent assay was used to determine cytokine and immunoglobulin levels.

Results This current study demonstrated that hUCMSCs attenuated both lung lymphocyte and eosinophil infiltration, and significantly decreased the concentration of Th2 cytokines interleukin-5 in splenocyte cultures.

Conclusions Human umbilical cord mesenchymal stem cells have the advantage of being easily harvested non-invasively and are capable of rapid proliferation, therefore an ideal material for stem cell-based immune therapies. The current study showed that fetal-type MSCs were able to suppress asthmatic symptoms efficiently, and its immunomodulatory effect resulted primarily from suppressing the Th2 pathway in the animal model. This study suggested that hUCMSCs could be an ideal candidate for cell-based therapies of asthma.

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Keywords Asthma · Allergic symptoms · Mesenchymal stem cell · MSC · Umbilical cord · Wharton's jelly · Murine · Mice

Introduction

Asthma is a chronic inflammatory disease caused by the loss of balance between the helper T cells, Th1 and Th2. Asthmatic symptoms result from antigens cross-linking antigen-specific IgE molecules which are bound to mast cells, leading to activation and degranulation of mast cells and the release of histamine, leukotrienes and other inflammatory substances. These effects recruit eosinophils regulated by interleukin (IL)-5, and finally cause airway damage and hyper-responsiveness [1].

Stem cells are undifferentiated cells characterized by the ability of self-renewing through cell division even after long periods of inactivity, and by their multipotency of becoming tissue-specific or organ-specific cells with special functions under certain conditions. Mesenchymal, or multipotent stem cells (MSCs) discussed in this current study are non-tissue-specific adult stem cells derived from various sources such as bone marrow, umbilical cord and umbilical cord blood, and are able to differentiate along multiple lineages [2–5]. In addition to MSCs regenerative ability, recent researches have shown that MSCs have the ability of immune regulation, even in a cross-species fashion [6–9]. Despite the fact that studies have shown that MSCs possess immunoregulatory effects both *in vitro* and *in vivo*, MSCs effects on asthma are not yet well-studied. This research is dedicated to understand the effects of immune regulation of MSCs in asthmatic mice. The results of this study support the potential of MSCs in asthma immunotherapy.

Furthermore, even though MSCs can be derived from bone marrow and adult organs, these sources face the disadvantages of their invasive procedure of isolation and the limited stem cell numbers. In this current study, we apply another source of MSCs, Wharton's jelly of umbilical cord (UC), which is not limited by the above-mentioned disadvantages, extending the possibility of MSCs clinical application. These human umbilical cord mesenchymal stem cells (hUCMSCs) are easily harvested with non-invasive procedure, can last long passages *in vitro*, and possess immunosuppressive properties [10]. Above all, hUCMSCs have already demonstrated stronger immunosuppressive effect than bone marrow-derived MSCs (BMMSCs) *in vitro* [11]. The hUCMSCs are thus an ideal material for future stem cell immune therapies for asthma.

Materials and methods

The hUCMSCs culture and supernatant collection

The hUCMSCs derived from Wharton's jelly of human umbilical cord were cultured in a flask with minimum essential media (MEM) α (Invitrogen, Life Technologies Corporation, Gaithersburg, MD, USA) containing 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin (P/S). TrypLE (Gibco, Life Technologies Corporation) was used to harvest cells. Oil red O stain and von Kossa stain were performed to verify that the cells preserved their differentiation capacity after long-term maintenance of the cell line (figure not shown).

OVA sensitization model of asthma in BALB/c mice

Female BALB/c mice at 8 weeks of age were used in the experiments. Care and handling of mice were according to the guidelines of the Animal Care Committee of Chang Gung University and the NIH Guidelines for the Care and Use of Laboratory Animals. Mice were separated into three groups: sensitized control group (S, $n = 10$), normal control group (N, $n = 5$) and hUCMSCs-injected experimental group (C, $n = 10$).

From day 1 to day 3 of the sensitization model, each BALB/c mouse was sensitized by intra-peritoneal (IP) injections with 50 μ g antigen ovalbumin (OVA) (Sigma, St. Louis, MO, USA) with 0.8 mg adjuvant aluminum hydroxide (Thermo, Rockford, IL, USA) in 200 μ l saline. Between each injection there was a 24-h interval. On day 14, OVA was again injected by IP injection, and after 2 h, mice were exposed to 2 % OVA in saline for 20 min for inhaling (IH) challenge. IH was performed every 3–4 days until day 28. The mice were killed under anesthesia on day 30. For normal control, OVA was replaced by saline throughout the whole procedure (Fig. 1).

Normal saline was given to normal control group throughout the sensitization model, and on day 21, saline without MSCs was injected via tail vein injection. On the same day, sensitized control mice were also injected with saline, and experimental groups were injected with 1×10^6 MSCs per mouse, both via tail vein injection.

On the day of killing, blood from venous plexus behind the eye was collected by a capillary tube. The serum was collected after centrifugation at 6,000 rpm for 10 min and then stored at -80°C . Bronchoalveolar lavage fluid (BALF) was obtained by inserting a cannula through a cut in the trachea and infusing 1 ml saline into bronchi with 3 washes per ml, and the fluid was recovered by aspirating with a syringe. A total of 3 ml of BALF was collected for each mouse (Fig. 1).

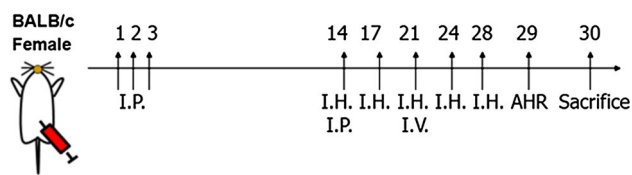


Fig. 1 The flow chart of OVA sensitization model of asthmatic BALB/c mice. Mice were sensitized and challenged with antigen OVA by intra-peritoneal injection (IP) and inhalation (IH), and normal control group was treated with saline. MSCs were introduced into the mice by intravenous injection (IV), while saline was injected in the control groups

Airway hyper-responsiveness (AHR)

Airway hyper-responsiveness of mice responding to increasing concentrations of aerosolized methacholine (Mch) was measured by placing the mice in plethysmography chambers (Buxco, Sharon, CT, USA). Continuous measurements of box pressure/time wave were calculated via a connected transducer and associated computer data acquisition system. Enhanced pause (Penh) value was the main indicator of airflow obstruction, which showed strong correlation with the airway resistance. After measurement of baseline Penh, mice were challenged with aerosolized Mch of increasing concentrations (10, 20, 30, and 40 mg/ml), and Penh value of each stimulation in each mice was recorded.

Lung cell infiltration

Cell pellets obtained from BALF was cytocentrifugated by Thermo Shandon Cytospin[®] 4 (Harlow Scientific, Arlington, Massachusetts, USA), and Liu's stain was performed to demonstrate the degree of cell infiltration. Liu A was stained for 45 s at room temperature, followed by staining Liu B for 90 s. BALF total WBCs were counted with a hemocytometer counting chamber, and cell differentials (monocytes, lymphocytes and eosinophil) were calculated from the percentage of each cell type taken from a count of at least 200 cells on a stained slide. OVA-sensitized mice were characterized by severe lung eosinophil infiltration, with eosinophils contributing up to 50 % of total lung white blood cells (WBCs).

OVA-stimulated splenocyte cultures

Each spleen was grounded and splenocytes in RPMI medium (Gibco, Life Technologies Corporation) were cultured in a 24-well plate. Each well contained 5×10^6 splenocytes and 1 ml RPMI with 100 µg/ml OVA. Six continuous days after OVA stimulation, supernatants were collected and stored at -80°C .

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was applied to analyze the levels of antibodies in serum. OVA-specific antibodies including IgG1, IgG2a and IgE were measured using a specific ELISA kit (BD Biosciences). The concentrations of OVA-specific IgG1 and IgG2a were quantified according to pre-existed serum standard and recorded with arbitrary unit, while the levels of IgE were demonstrated by optical density (OD) at 450 nm.

Statistical analysis

The results were statistically analyzed with GraphPad Prism 5 software. Mann–Whitney *U* test and two-tailed *T* test with 95 % confidence interval were applied to find whether significant difference exists and to calculate *p* value for each set of data. Data were presented by graphs demonstrating mean $\pm$ SEM.

Results

Lung inflammation was attenuated by hUCMSCs

Airway hyper-responsiveness is an important feature of asthma that correlates to the severity of bronchospasm triggered by an antigen, in this case, OVA. Compared to the AHR level in sensitive control group (mean $\pm$ SEM 3.05 ± 1.24), the AHR level in MSCs-treated group (1.69 ± 0.46) showed a relative reduction but without significant difference (Fig. 2).

On the other hand, the number of total WBC in BALF cell count directly indicates the severity of lung lymphocyte infiltration. MSCs-injected group (3.88 ± 0.55) showed a decrease in total number of lung WBCs, while greatest total WBC level was observed in sensitized control group (5.76 ± 0.99), suggesting an attenuation in lung lymphocyte infiltration in MSCs-treated mice (Fig. 3a). Additionally, IL-5 level in BALF also had a downward trend in MSCs-injected group, although the final statistical result of IL-5 levels between the sensitized group and MSCs-injected group were not significant (figure not shown).

Also, the eosinophil percentage of total WBC count was highest in sensitized control (52.63 ± 5.14), and the percentage of MSCs-injected group (19.91 ± 4.30) decreased significantly comparing to the sensitized control ($p < 0.01$). In fact, the eosinophil percentage of MSCs-injected group was reduced to a level approaching that of normal control group (Fig. 3b), and the eosinophil cell number in the lung samples of MSCs-injected mice was significantly decreased as well ($p < 0.01$) (Fig. 3c).

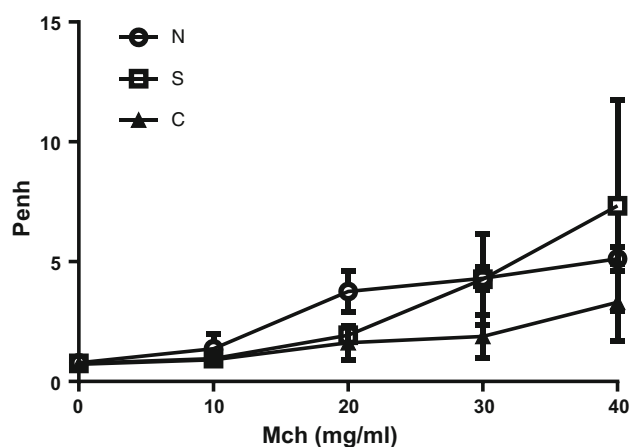


Fig. 2 Airway hyper-responsiveness (AHR) measured as Penh. Higher level of Penh indicated more severe bronchospasm. Mice were divided into normal group (N), sensitized group (S) and hUCMSCs-treated group (C). The AHR levels in MSCs-treated group and sensitive control group showed an overall reduction but without significant difference

No significant difference in IgG1, IgG2a and IgE levels of the MSCs-injected mice was detectable comparing to the levels of sensitized control mice

IgE is the main immunoglobulin mediating allergic responses, and high level of IgE indicates a recent allergic attack. IgG1 is the immunoglobulin related to Th2-type reaction, while Ig2a is related to Th1-type reaction. In this study, however, it was observed that the level of IgG1 in MSCs-treated group ($27.3 \pm 6.1 \times 10^3$ KU/ml) only showed a slight decrease compared to the IgG1 level of sensitized control (28.0 ± 5.7), while the level of IgG2a in MSCs-treated group ($0.62 \pm 0.11 \times 10^3$ KU/ml) decreased significantly compared to that of sensitized control (1.01 ± 0.31) (Fig. 4a, b). On the other hand, the levels of IgE did not show significant difference between each group (Fig. 4c).

The main Th2 cytokine IL-5 was significantly reduced in MSC-injected mice

Cytokines in the supernatants of splenocytes stimulated with OVA were measured with ELISA. In MSC-treated group, IL-5 showed significant reduction in MSC-treated mice ($1,983.3 \pm 512.6$ pg/ml) comparing to that of sensitized control ($5,419.8 \pm 1,038.2$, $p < 0.01$). Nonetheless, the levels of IL-4 (74.7 ± 45.9), IL-10 ($2,227.8 \pm 489.0$) and IL-13 ($1,450.8 \pm 884.5$) in MSC-treated mice were

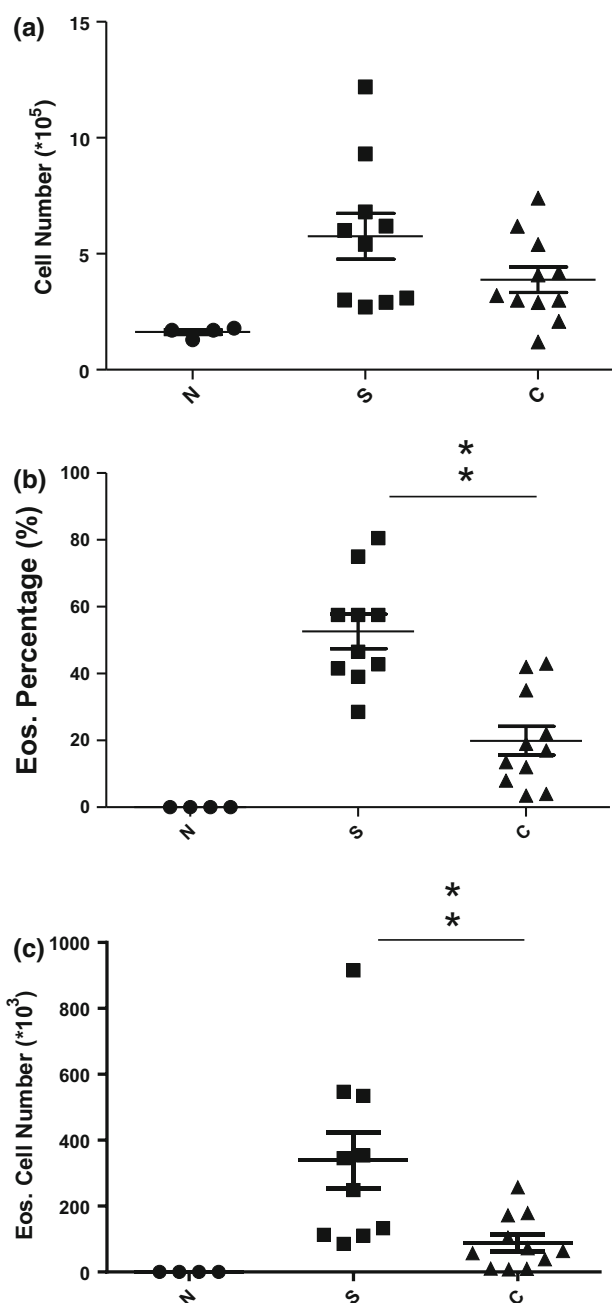


Fig. 3 **a** Total WBCs number, **b** the percentage of eosinophils of lung WBC, and **c** the total number of lung eosinophils in normal group (N), sensitized group (S) and hUCMSCs-treated group (C). There was no significant difference of total lung WBCs number between MSC-treated and sensitized control. The eosinophil percentage was significantly decreased in MSCs-injected group comparing to the sensitized control ($p = 0.0079$). The total cell number of eosinophils in the lungs of MSCs-treated mice also reduced significantly ($p = 0.0046$). WBC white blood cells. * $p < 0.05$ and ** $p < 0.01$ when compared to the sensitized control group

comparable to the IL-4 (286.3 ± 95.5), IL-10 ($3,160 \pm 360.2$) and IL-13 ($2,032 \pm 709.1$) levels of sensitized control (Fig. 5).

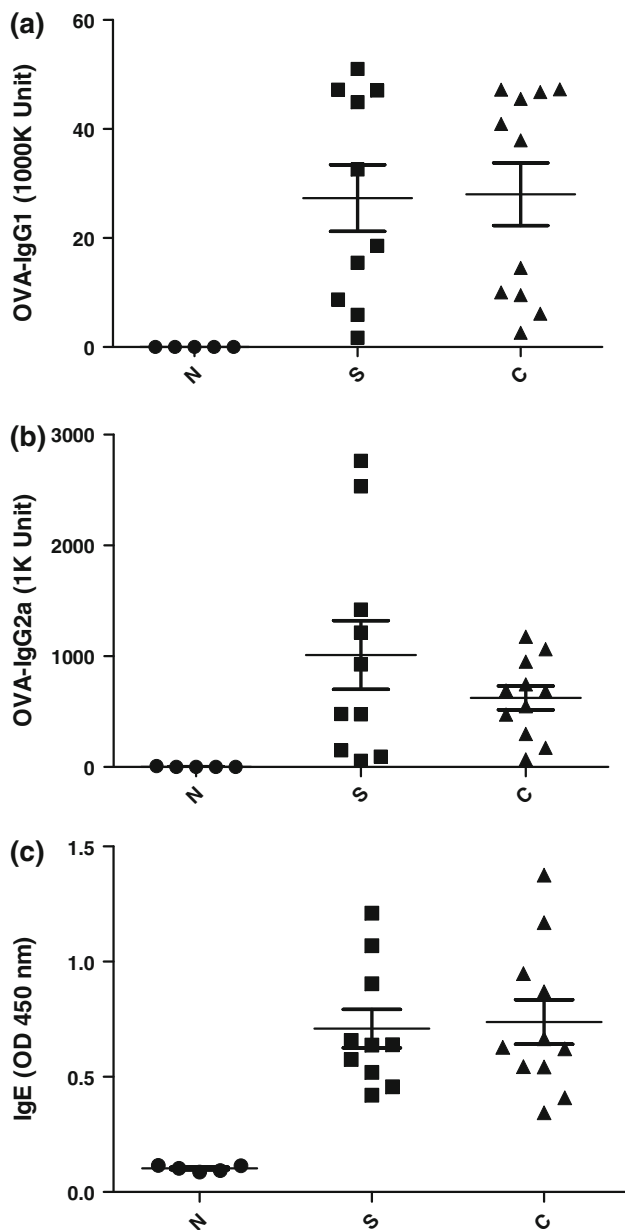


Fig. 4 The concentration of OVA-specific Th2-related antibody **a** IgG1 and Th1-related antibody, **b** IgG2a in astronomical unit (AU), and **c** OVA-specific IgE in 40-fold diluted serum. Pooled sera from OVA-sensitized mice were used to create OVA-specific IgG1 and IgG2a standard curves for quantification of the IgG1 and IgG2a levels based on optical density (OD) measurement at 450 nm. The IgG1 and IgG2a levels were recorded with arbitrary unit, and IgE levels with OD₄₅₀ readings. The level of IgG1, IgG2a and IgE in MSCs-treated group was comparable to that of sensitized control. No detectable antibodies were present in normal control

Discussion

Recent studies have shown that hUCMSCs have stronger suppressive effects than BMMSCs on peripheral blood mononuclear cell (PBMC) proliferation in vitro [11, 12].

As for in vivo experiments, a previous research has demonstrated that either allogeneic or human BMMSCs have immunosuppressive effect on OVA-sensitized asthmatic mice [9, 13]. Therefore, this current study seeks to explore the effectiveness of the immunosuppressive response of hUCMSCs in vivo, and its possible mechanism.

There are several similarities between the in vivo experiments conducted with BMMSCs and this current study. Both discover reduced lung inflammatory status, significant decrease in eosinophilic Th2 inflammation and IgE serum concentrations [9, 14–18]. Nevertheless, the BMMSCs research in vivo did not demonstrate the significant decrease in the number of eosinophil of BALF as this current study does. Moreover, this current study further investigates the role of IgG1, IgG2a and ILs could have in immunosuppressive properties of MSCs. In addition, our study indicates that a reduction in AHR, measured as Penh, is associated with decreased serum OVA-specific immunoglobulins production and with reduced eosinophil lung infiltration in MSCs-injected OVA-sensitized mice. Also, decreased IL-5 production in splenocyte culture correlates with reduced eosinophil infiltration in MSCs-treated mice, suggesting that human MSCs are capable of suppressing the asthmatic symptoms in OVA-sensitized mice. The data of IL-13, however, is comparable between the control and experimental group, which might be reasonably explained by the compensation of IL-13 toward IL-4 [19, 20]. In short, this current study implies the possible mechanism of how MSCs exert its immunosuppressive effect: by modulating the Th2 pathway

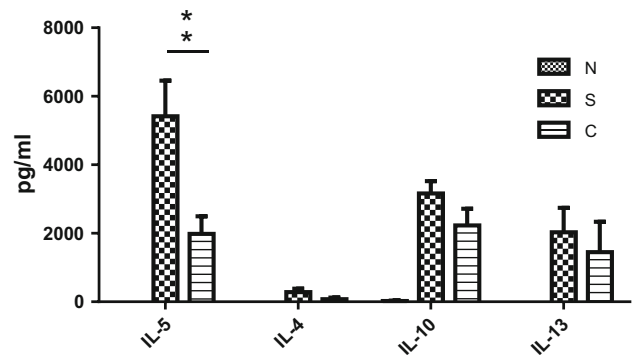


Fig. 5 The IL-5, IL-4 and IL-10 concentrations in OVA-stimulated splenocytes culture. Splenocytes were stimulated with 100 µg/ml OVA in the medium for 6 days, and then supernatants were collected for cytokine concentration assessment. A significant declined IL-5 level was present in MSC-treated group compared to sensitized control ($p = 0.0082$). The IL-4, IL-10 and IL-13 concentrations were at comparable levels between sensitized control and MSC-treated group. No detectable cytokines were present in normal control. IL interleukin. * $p < 0.05$ and ** $p < 0.01$ when compared to the sensitized control group

in the animal model. Although it has been shown that MSCs exert strong suppressive effects on Th1 cell differentiation and effector function in vitro, very few studies have examined the effects of MSCs on immune-mediated diseases which are dominated by Th2 cell responses [21].

Currently, MSCs have been widely applied in tissue engineering research and considered as appealing due to their easy accessibility and non-invasive procedure [22]. Not only laboratory researches but also clinical trials have been conducted to examine the treatment of autoimmune disease with MSCs [23]. In fact, preclinical studies suggest that MSCs are capable of successfully ameliorating autoimmune diseases such as systemic lupus erythematosus (SLE) [24], rheumatoid arthritis [25] and experimental autoimmune encephalomyelitis (EAE) [26]. Since hUCMSCs are more accessible and non-invasive to obtain clinically, the cells could thus have more potential to benefit patients if applied successfully to clinical use.

On the other hand, the cross-species fashion of immunosuppression observed in this current study can be a clue to the molecular mechanisms of the immunosuppressive effect of MSCs. Since human MSCs less likely regulate the immune function in mice in a cell-mediated fashion, soluble factors that possess immunosuppressive properties such as certain metabolites might be the main contributors of the immunomodulatory effects. Although remaining controversial, there are researches suggesting that MSCs express their immunosuppressive effects by secreting soluble molecules such as nitric oxide, PGE₂, IL-6 and many other mediators [11, 27–33]. Even though the exact molecular mechanism of MSCs immunosuppressive effect is not yet clear, in this current study of asthmatic mice, we hypothesize that the pathological condition in vivo induces MSCs to secrete substances that reduce serum IgG and IL-5 levels.

In summary, this study demonstrated that hUCMSCs alleviate asthmatic symptoms a murine model of asthma. Even though the cross-species immunosuppressive effect of MSCs is not yet thoroughly studied, this phenomenon implies that MSCs might produce certain soluble substances such as metabolites which probably result in their immune regulatory properties. In-depth study of this suppressive effect should lead to more understanding of the properties of MSCs and to the expansion and acceleration of its clinical use.

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Compliance with ethical standards

Conflict of interest No financial support or other benefits from commercial sources was provided for this work.

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