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Effect of Inducible Co-Stimulatory Molecule siRNA in Cerebral Infarction Rat Models

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Background: T cell-induced inflammatory response and related cytokine secretion at the injury site may participate in the pathogenesis of cerebral infarction. Recent studies established inducible co-stimulatory molecule (ICOS) as a novel T cell-related factor for its activation and functions. We thus investigate the role of ICOS in cerebral infarction.

Material/Methods: The siRNA of ICOS was first used to suppress the gene expression in cultured lymphocytes. An *in vivo* study was then performed by intravenous application of ICOS siRNA in cerebral infarction rats. Survival rates, neurological scores, serum tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-17 levels were observed.

Results: The expression of ICOS in cultured lymphocytes was significantly suppressed by siRNA. In the *in vivo* study, the application of siRNA effectively lowered mortality rates of rats, in addition to the improvement of neurological behaviors and amelioration of cerebral tissue damage. Serum levels of TNF-α, IL-1 and IL-17 were all significantly suppressed after siRNA injection.

Conclusions: ICOS siRNA can protect brain tissues from ischemia injuries after cerebral infarction, improve limb movement and coordination, lower the mortality rate of rats, and inhibit T cell-induced cytokines. These results collectively suggest the potential treatment efficacy of ICOS siRNA against cerebral infarction.

MeSH Keywords: Cerebral Infarction • Hyperlipoproteinemia Type IV • Rats, Inbred BUF

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Background

Cerebral infarction, or cerebral ischemia, is a major cause of death and disability in adults, causing a heavy burden for the health system and patients' families. Studies have pointed out various possible mechanisms in the occurrence of cerebral infarction [1–4]. Inflammatory response is an important pathological mechanism of cerebral ischemia. This is supported in animal models, as inflammation aggravated brain tissues even after the primary focal cerebral infarction [5–7]. Detailed observation revealed that a series of T cell-induced inflammatory responses occurred in the peripheral region of ischemia tissues, and the severity of inflammatory response is positively related with neurological damage [8,9]. Meantime, T cell-secreted cytokines also play a role in the inflammatory injury after cerebral ischemia. Numerous *in vitro* and *in vivo* studies have indicated tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-17 as important factors in the pathogenesis of infraction-induced cerebral tissue damage, as their expression levels are elevated in both ipsilateral brain tissues and peripheral blood serum [10–14].

As a classical co-stimulatory signal molecule, inducible co-stimulatory molecule (ICOS) plays a critical role in the activation of T cell immune efficacy. Specifically, T cells can acquire certain co-stimulatory signals by the interaction between ICOS and its ligands, during the antigen-specific interaction between T cells and dendritic cells or antigen specific B cells [15,16]. Studies have proved the role of co-stimulatory signals induced by the binding between ICOS and its ligands in regulating various cellular events such as T cell activation/proliferation and secretion of cytokines, including TNF-α, IL-1, and IL-17 [17,18]. Recent studies have demonstrated the connection between ICOS-ligand signal pathways and autoimmune disease or inflammatory response [18,19]. Further studies revealed an important role of ICOS in the differentiation and maintenance of Th cells [17–19]. Data on the role of ICOS in cerebral infarction, however, is still lacking. Thus, we designed ICOS-specific siRNA to inhibit the *in vitro* gene expression, and then applied siRNA in cerebral infarction rat models. Mortality rates and neurological scores, along with serum cytokine levels, were observed to reflect the role of ICOS in the pathogenesis of cerebral ischemia-related tissue damages.

Material and Methods

Culture of lymphocytes

After anesthesia, SD rats were sacrificed by decapitation. The peritoneal cavity was opened after skin sterilization. The spleen was removed, ground, filtered, and rinsed by Hank's solution. The collected spleen cell suspension was then centrifuged at 2000 rpm for 3 min. After discarding the supernatant, 30 mL of erythrocyte lysate was added for 5-min incubation. After the complete lysis of erythrocytes, the mixture was re-centrifuged at 2000 rpm for 3 min to discard erythrocyte debris. After washing by Hank's solution, lymphocytes were re-suspended in RPMI-1640 medium (Yubo BioTech, China) with 10% fetal bovine serum (FBS). The final concentration of cell suspensions was adjusted to 8~10×10^6 per mL. Cells were cultured using DMEM culture medium (Yubo BioTech, Shanghai, China) containing 5 mM D-glucose at 37°C with 5% CO2.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the Second Xiangya Hospital of Central South University.

siRNA transfection

One day before the transfection, cultured lymphocytes were inoculated into Petri dishes without antibiotics. Cells with 30% confluence were transfected with siRNA of ICOS or non-specific (NS) controlled siRNA (Santa Cruz, USA) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. In brief, diluted liposome solutions were mixed with siRNA, followed by 5-min incubation at room temperature. The mixture was then added into the cultured dish, which was filled with 5 mL of serum-free DMEM culture medium. After 6 h, the medium was changed to DMEM with 10% serum.

2 RT-PCR for ICOS expression assay

Four-eight hours after transfection, cells (5×10^6) in all groups were collected and extracted for total RNA using Trisol kits (Invitrogen, USA) following the manufacturer's instructions. ICOS-specific primers were designed by Primer 5.0 software with the following sequences: ICOS-F, 5'- GUG CAC GAC UCA AUA TA-3'; ICOS-R, 5'- TTC ACG UGC UGA CGC AG-3'; β-actin-F, 5'- GGT GTG ATG GTG GGT ATG GGT-3'; β-actin-R, 5'- CTG GGT CAT CTT TTC ACG GT-3'. Quantitative RT-PCR was carried out using SYBR PCR kits (Invitrogen, USA) following the manufacturer's instructions with the following conditions: pre-denature for 5 min at 94°C; 30 cycles of amplification, each containing 1-min denature at 94°C, 1-min annealing at 60°C, and 3-min elongation at 72°C; ending with 5-min elongation at 72°C. Relative expression level of mRNA was calculated using $2^{-\Delta\Delta Ct}$ method based on the standard curve.

Rat model of cerebral infarction

Thirty SD rats (15 males and 15 females; body weight= 280~300 g) were obtained from the experimental animal center of the Chinese Academy of Science (Shanghai). The dried blood clots from SD rats were ground, filtered, and sterilized. Blood clots were suspended into a 200-mg/mL mixture in saline before use. Rats were anaesthetized by 3% pentobarbital (1 mL/100 g) and fixed on the table. A middle anterior neck

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incision was made to expose the left common carotid artery, followed by the separation of internal/external carotid artery. A metal clip was used to temporally block the common carotid artery and a 0.5-mL thrombus mixture was then injected via a catheter inserted into the left external carotid artery, which was clipped immediately after the infusion. The common carotid artery was opened to let the thrombus enter the cerebral artery, causing multifocal cerebral infarction. A parallel sham group was also prepared with the same surgical procedure and saline injection. Rats after surgery were singly housed and given antibiotics.

Neurological observation

One day after the surgery, all rats were examined for neurological behaviors and were graded as: Grade 0, normal activity; Grade I (mild), able to walk but with decreased body resistance (animals can be pushed back for 10~15 cm); Grade II (moderate), can walk but with cycled gait pattern; Grade III (severe), can stand but not walk. Animals with Grade I or above were defined as having cerebral infarction. All rats meeting these criteria were randomly divided into model group, ICOS siRNA treatment group and siRNA control group. Three days after the surgery, 0.2 mL saline, ICOS siRNA, or NS controlled siRNA were applied via intravenous injection. Mortality rates of all animals were continuously monitored and neurological behaviors were observed 2 weeks after the injection.

Enzyme-linked immunosorbent assay (ELISA)

Venous blood samples were collected and extracted for lymphocytes, which were cultured in DMEM medium containing 10% FBS. After 48 h, supernatants of cell culture were collected and tested for TNF-α, IL-1, and IL-17 levels using ELISA kits (BD Corp., USA) following the manufacturer's instructions. All assays were performed in triplicate.

Statistical analysis

The SPSS 17.0 software package was used to analyze all collected data, which are presented as mean ± standard deviation (SD). Between-group comparisons were performed by t test. Multiple group comparisons were applied using 1-way analysis of variance (ANOVA). A statistical significance was defined when $p<0.05$.

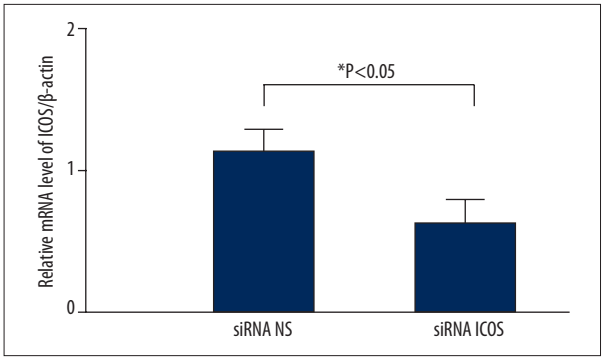

Figure 1. Expression of ICOS mRNA in culture lymphocytes. Relative mRNA levels of ICOS were significantly lowered in siRNA ICOS-transfected cells compared to those in cells transfected with NS-controlled siRNA. * p<0.05, t test.

Results

In vitro expression assay of ICOS

The transfection of cultured lymphocytes by ICOS siRNA significantly suppressed the expression of ICOS, as suggested by the RT-PCR study in which ICOS-siRNA transfection decrease the expression of the target gene by more than 40% (Figure 1).

Mortality rates of cerebral infarction rats

As shown in Table 1, 6 out of 20 (30%) rats died 2 weeks after the application of saline, 4 out of 20 (20%) rats that received NS siRNA died, and only 1 out of 20 (5%) rats died that received ICOS siRNA. Between-group comparison showed statistical significance ($p<0.05$, 1-way ANOVA). These results suggest the potential effect of ICOS siRNA in decreasing the mortality rate of cerebral infarction.

Neurological behavior of cerebral infarction rats

Two weeks after the injection, there were 4, 10, and 6 rats in the model group being classified into Grade I, II, and III, respectively. In the NS siRNA group, the numbers were 8, 8, and 4. When rats received ICOS siRNA, however, the number of Grade III rats decreased to zero, and the number of Grade I

Table 1. Mortality rate of cerebral infarction rats (2 weeks after siRNA treatment).

Group	N	Mortality No	Mortality rate (%)
Sham	20	0	0
Model	20	6	30
ICOS siRNA	20	1	5
NS siRNA	20	4	20

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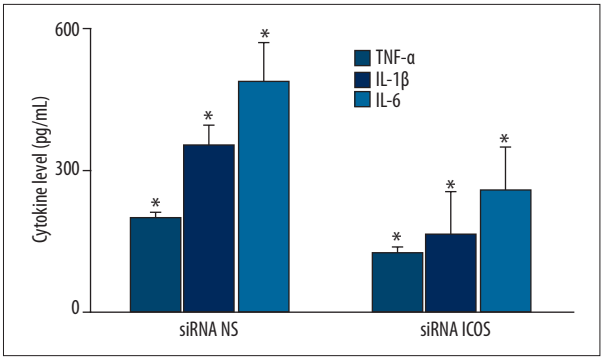

Figure 2. Serum levels of TNF-α, IL-1, and IL-17. Cytokine levels were quantified by ELISA and compared between NS siRNA- and ICOS siRNA-injected rats. All 3 cytokines (TNF-α, IL-1β, and IL-17) showed depressed secretion after the application of ICOS siRNA. * p<0.05, t test.

animals increased to 13. All these differences were of statistical significance (1-way ANOVA, p<0.05), showing that siRNA can improve the neurological behavior and ameliorate motor deficits of cerebral infarction.

Cytokine secretion of cerebral infarction rats

As shown in Figure 2 and Table 2, the application of ICOS siRNA can significantly decreased the level of TNF-α, IL-1, and IL-17 in peripheral blood by more than 50% (p<0.05, t test), suggesting the role of ICOS in the induction of secondary inflammation after cerebral ischemia.

Discussion

A major challenge of cerebral infarction research is the limitation of replicable animal models. A reliable and consistent methodology to induce cerebral ischemia is therefore of critical importance for elucidating the pathology of cerebral ischemia. Our animal models showed limb paralysis on the contralateral side with various severities, consistent with multi-focal cerebral infarction in clinics [5–9]. All animals that received the thrombosis infusion reached neurological deficit grade I or above (Table 3), suggesting the efficacy of the surgery.

Stress response can be activated after acute cerebral infarction for the further induction of the hypothalamus-pituitary-adrenal gland axis, thereby causing the secretin of cortical hormones for modulating T cell distribution and function [12–16]. ICOS and its ligand, on the other hand, exert critical functions on T cell-related immune response. In this study, ICOS-specific siRNA was for the first time synthesized and utilized in rat cerebral infarction models. The efficacy of siRNA can be confirmed by *in vitro* lymphocytes (Figure 1). Further *in vivo* testing showed that, compared to those that received NS-controlled siRNA or saline, cerebral infarction rats after ICOS siRNA injection had significantly lowered mortality rate (Table 1) and improved motor behaviors, as shown by neurological grades (Table 3). Our results suggest that the suppression of ICOS gene expression can effectively improve the limb movement and motor coordination of cerebral infarction rats, lower the mortality rate, and ameliorate neural tissue damage.

A further investigation revealed a possible mechanism of ICOS by showing that the application ICOS siRNA decreased the secretion of TNF-α, IL-1, and IL-17 from Th1 lymphocytes (Figure 2). These data suggest that ICOS is potentially involved in the inflammatory response after primary cerebral ischemia. Various studies have shown that an acute inflammatory response occurred after the onset of acute cerebral infarction, resulting in the elevated expression of pro-inflammatory cytokines, including TNF-α, IL-1, and IL-17 [17–19]. Other studies demonstrated the efficacy of multiple neural protective agents such as edaravone in decreasing serum levels of pro-inflammatory factors

Table 2. Cytokine secretion levels in cerebral infarction rats.

Group	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-17 (pg/mL)
ICOS siRNA	186.0±9.3	320.1±20.6	476.3±86.2
NS siRNA	87.7±7.5	152.3±62.3	275.3±98.7

Table 3. Animal numbers in each behavioral grade after siRNA injection.

Group	N	Grade I	Grade II	Grade III
Sham	20	0	0	0
Model	0	4	10	6
ICOS siRNA	0	8	8	4
NS siRNA	0	13	7	0

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and treating cerebral ischemia [12–16]. Our results revealed a similar role of ICOS siRNA in suppressing pro-inflammatory cytokines and in inhibiting inflammatory response.

Both *in vitro* and *in vivo* studies have shown the critical function of ICOS and its ligands in both formation and function of T cells, Th1, and Th17 cells [20–23]. Further studies showed the facilitation of ICOS signal pathway in the secretion of Th1-related cytokines (IFN-α, TNF-α, and IL-1) and Th17-related cytokine (IL-17). ICOS gene knockout mice showed no immunity against bacterial or viral inflammation, accompanying with deficits in Th cell-related immune response [24], thereby attributing the Th cell immunity to the ICOS signal pathway. Studies also showed that the absence of ICOS inhibited the progression of autoimmune disease or graft rejection [25,26]. All these studies show the importance of ICOS in cellular immunity.

Conclusions

In summary, this study for the first time indicated the potential treatment efficacy of ICOS siRNA in cerebral infarction, possibly via the inhibition of Th1 and Th17 cytokine secretion. In clinical practice, only thrombolytic therapy at the super-acute period, urokinase and streptokinase have been approved for treating cerebral infarction. All these methods, however, only exert satisfactory efficacy within 3~6 hours of the occurrence of cerebral ischemia. Our studies showed that the application of ICOS siRNA 3 days after cerebral infarction can still improve motor behavior and decrease mortality rates, thereby providing a novel candidate for brain ischemia treatment.

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