Deletion of Angiotensin II Type 2 Receptor Attenuates Protective Effects of Bone Marrow Stromal Cell Treatment on Ischemia–Reperfusion Brain Injury in Mice

Jun Iwanami, MS; Masaki Mogi, MD, PhD; Jian-Mei Li, MD, PhD; Kana Tsukuda, BS; Li-Juan Min, MD, PhD; Akiko Sakata, MD; Teppei Fujita, MD; Masaru Iwai, MD, PhD; Masatsugu Horiuchi, MD, PhD

Background and Purpose—Protective effects of bone marrow stromal cells (MSCs) on ischemic brain damage have been highlighted. We examined the possibility that deletion of AT₂ receptor could attenuate the cerebroprotective effects of MSC using AT₂ receptor-deficient mice (Agtr2²⁻) and the effect of selective AT₁ receptor blocker.

Methods—Wild-type mice (Agtr2²⁺) were subjected to 3 hours of focal brain ischemia followed by reperfusion (ischemia–reperfusion injury). Simultaneously, Agtr2²⁺-MSC, Agtr2²⁻-MSC, or saline was injected through the tail vein.

Results—Survival rates at 6 days after ischemia–reperfusion injury were as follows: approximately 50% in saline-injected mice, 80% in Agtr2²⁺-MSC-injected mice, and 20% in Agtr2²⁻-MSC-injected mice. Neurological deficit after ischemia–reperfusion injury was improved in Agtr2²⁺-MSC-injected mice, but not in Agtr2²⁻-MSC-injected mice. After 48 hours of ischemia–reperfusion injury, brain infarct size was reduced in Agtr2²⁺-MSC-injected mice, but not in Agtr2²⁻-MSC-injected mice. Moreover, brain edema was significantly ameliorated in Agtr2²⁺-MSC-treated mice but not in Agtr2²⁻-MSC-treated mice. Furthermore, the increase in mRNA expression of tumor necrosis factor-α and monocyte chemoattractant protein-1 in the ischemic brain was less in Agtr2²⁺-MSC-treated mice in the ipsilateral site, but was similar in the contralateral hemisphere. Tumor necrosis factor-α level was increased in both the contralateral hemisphere and ipsilateral hemisphere of Agtr2²⁻-MSC-treated mice. In contrast, monocyte chemoattractant protein-1 levels tended to increase Agtr2²⁻-MSC-treated mice without a significant difference. Treatment of MSC with an AT₁ receptor blocker, valsartan, significantly improved survival rates in Agtr2²⁻-MSC-injected mice.

Conclusions—These results suggest that AT₂ receptor signaling in MSC attenuated brain damage and neurological deficit (deleted). (Stroke. 2008;39:2554-2559.)

Key Words: angiotensin II receptor ■ bone marrow stromal cell ■ brain edema ■ inflammatory cytokines ■ stroke

Stroke is one of the leading causes of death and quality-of-life impairment due to neurological deficit; however, radical treatment for stroke is limited. Recently, cellular therapy has been focused on as a new therapeutic approach to restore injured neurons in the chronic stage and to protect neurons from ischemic–reperfusion damage in the acute phase of stroke using bone marrow stromal cells (MSCs), neural stem cells, hematopoietic stem cells, and umbilical cord blood. MSCs are characterized by the ability to self-renew in a number of nonhematopoietic tissues and by their multipotentiality for differentiation into various tissues such as fibroblasts, bone, muscle, and cartilage. In addition, MSC can differentiate into cells with some characteristics of neurons and astrocytes after being implanted into the central nervous system in vivo. MSCs can also protect neurons by secretion of growth factors and cytokines into the brain. Subsequently, many previous reports have demonstrated that MSC transplantation improves functional recovery after stroke. However, the detailed mechanism of the neuroprotective function of MSC after stroke is totally unknown.

Recent large clinical trials such as the LIFE and MOSES studies indicated that blockade of the renin–angiotensin system is effective to prevent a first or recurrent stroke beyond blood pressure-lowering. However, the detailed molecular mechanisms of preventing the onset of such pathological conditions are still an enigma. Angiotensin II is the principal vasoactive substance of the renin–angiotensin system, having a variety of physiological actions, including vasoconstriction, aldosterone release, and cell growth. Angiotensin II binds 2 major receptors, the angiotensin II type 1.
(AT₁) receptor and type 2 (AT₂) receptor. Although the majority of angiotensin II actions are mediated through the AT₁ receptor, accumulating evidence has suggested that the AT₂ receptor in general not only opposes the AT₁ receptor, but also has its own effects independent of an interaction with AT₁ receptor signaling. We reported that activation of the AT₂ receptor attenuated brain injury partly due to a reduction of oxidative stress in the ischemic brain and an increase in cerebral blood flow in the penumbral region in mice subjected to middle cerebral artery (MCA) occlusion. Moreover, we demonstrated that AT₂ receptor signaling also enhanced neural differentiation and the repair of damaged DNA by induction of a neural differentiating factor, methyl methanesulfonate-sensitive 2 (MMS2), which is one of the ubiquitin conjugating enzyme variants. Recent studies have also demonstrated the possibility that stimulation of AT₂ receptors may promote cell differentiation and regeneration in neuronal tissue. Li et al reported that AT₂ receptor stimulation supported neuronal survival and neurite outgrowth in response to ischemia-induced neuronal injury. Moreover, Gallo-Payet et al demonstrated that angiotensin II induces neural differentiation and neurite outgrowth through mitogen-activated protein kinase or nitric oxide through AT₂ receptor activation and is involved in cerebellar development. This accumulating evidence indicates that AT₂ receptor signaling acts as a crucial cerebroprotective factor after stroke.

All components of renin–angiotensin system are detected in cultured MSC by reverse transcriptase–polymerase chain reaction and flow cytometry. The bone marrow renin–angiotensin system has been reported to contribute to regulation of hematopoiesis, especially in erythropoiesis. The AT₂ receptor is reported to be widely expressed in the fetal–placental unit, but is observed at low levels in adult tissues and is re-expressed in some pathological conditions, indicating an important role of AT₂ receptor activation in tissue regeneration. However, the roles of the AT₂ receptor in MSC transplantation after stroke have never been investigated. Here, we examined the possibility that stimulation of AT₂ receptor signaling in MSC could contribute to brain protection in a mouse focal brain ischemia–reperfusion model induced by MCA occlusion.

Materials and Methods

Animals
Adult male AT₂ receptor-deficient mice (Agtr2⁻/⁻; based on C57BL/6J strain) and wild-type mice (Agtr2⁺/⁺; C57BL/6J) at 10 to 12 weeks old were used in this study. Mice were provided by CLEA; Tokyo, Japan. There was no difference in blood pressure between these mice (Supplemental Figure I, available online at http://stroke.ahajournals.org). The experimental protocol was approved by the Animal Studies Committee of Ehime University.

Middle Cerebral Artery Occlusion and Reperfusion
Focal cerebral ischemia was induced by occlusion of the left middle cerebral artery with a modified intraluminal filament technique as described previously. For reperfusion injury, the nylon filament was removed from the common carotid artery 3 hours after MCA occlusion.

Preparation of Bone Marrow Stromal Cells
Bone marrow cells were isolated from 6 crushed bones (bilaterial of tibias, femurs, and iliac bones) in each experiment and placed in polystyrene cell culture dishes (Corning, NY). After 24 hours of incubation, nonadherent cells were removed, and attached cells were incubated for 24 hours with or without a selective AT₁ receptor blocker, valsartan (provided by Novartis Pharma AG) at a dose of 10⁻⁵ mol/L. MSC (2.0×10⁵ cells suspended in 200 μL) were injected through the tail vein after diluting in phosphate-buffered saline (200 μL) immediately after the reperfusion. Hemodynamic change such as cerebral blood flow and blood pressure were not changed after MSC injection.

Neurological Score
Neurological deficit was evaluated 24 hours after MCA occlusion using the neurological scores developed by Huang et al.

2,3,5-Triphenyltetrasodium Chloride Staining
To evaluate the ischemic area in the brain, the extracted brain was sliced into 7 coronal sections with 1-mm thickness and stained with 2% 2,3,5-triphenyltetrasodium chloride. Ischemic size and volume were determined as the percentage of 2,3,5-triphenyltetrasodium chloride-unstained area in the total area.

Brain Water Content and Electrolytes
Brain water content was measured by the wet/dry weight method as described previously. Brains were weighed wet and then oven-dried at 100°C for 24 hours and reweighed. Brain water content (%) was calculated as (wet weight–dry weight)/wet weight×100.

Progenitor Colony Formation Assays
Fresh Agtr2⁻/⁻ and Agtr2⁺/⁺ whole marrow cells were assessed by in vitro methylcellulose-based colony-forming unit assay (MethoCult; StemCell Technologies Inc, Vancouver, BC, Canada). Cells were plated at a concentration of 2×10⁴ cells per plate. After 2 weeks, the colonies were scored using a dissecting microscope at ×20 magnification.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction Method
Real-time quantitative reverse–transcription polymerase chain reaction was performed with a SYBR green i kit (MJ Research, Inc, Waltham, Mass). The polymerase chain reaction primers are described in extended “Materials and Methods” of the supplemental file.

Statistical Analysis
All values were expressed as mean±SEM. Data were analyzed by Kruskal-Wallis H test. If a statistically significant effect was found, Mann-Whitney rank sum test was used. Survival rate was analyzed by log-rank test. A value of P<0.05 was considered statistically significant.

Results
Lack of AT₂ receptor in marrow stromal cells failed to improve survival rate and neurological deficit in mice with ischemia–reperfusion injury.

Marrow stromal cells prepared from C57BL/6J mice (Agtr2⁻/-MSC), angiotensin II type 2 receptor-deficient mice (Agtr2⁻/-MSC) or saline as a control was injected through the tail vein immediately after reperfusion. As shown in Figure 1A, saline-injected mice exhibited approximately 50% survival rate after ischemia–reperfusion injury 6 days after MCA occlusion, whereas approximately 80% of mice with Agtr2⁻/-MSC injection survived after ischemia–reperfusion injury. Interestingly, Agtr2⁻/-MSC-injected mice showed a marked
Neurological deficit was evaluated by neurological examination for signs such as hemiplegia, loss of balance, and no spontaneous movements. Mice exhibited neurological deficits after MCA occlusion for 2 days. Brain sections were obtained 24 and 48 hours after ischemia–reperfusion injury. A, 2,3,5-triphenyltetrasodium chloride staining of ischemic areas. B, Quantitative analysis of ischemic area of 24 or 48 hours after I/R injury determined by 2,3,5-triphenyltetrasodium chloride staining (expressed as a percentage of total area). Brain sections are numbered from frontal (section 1) to caudal (section 7), and stroke volume was evaluated. n=5 to 8 for each group. *p<0.05 versus saline. \( p<0.05 \) versus \( Agtr^{2+} \). Values are mean±SEM.

Figure 2. Stroke size and volume after ischemia–reperfusion (I/R) injury. A, 2,3,5-triphenyltetrasodium chloride staining of brain sections of 48 hours after I/R injury. B, Quantitative analysis of ischemic area of 24 or 48 hours after I/R injury determined by 2,3,5-triphenyltetrasodium chloride staining (expressed as a percentage of total area). Brain sections are numbered from frontal (section 1) to caudal (section 7), and stroke volume was evaluated. n=5 to 8 for each group. *p<0.05 versus saline. \( p<0.05 \) versus \( Agtr^{2+} \). Values are mean±SEM.

Lack of AT1 Receptor in Marrow Stromal Cells Failed to Attenuate Brain Edema With Ischemia–Reperfusion Injury

Because it is reported an increase in the blood–brain barrier permeability to sodium occurred from 12 to 48 hours after MCA occlusion, we next evaluated brain edema in the 48 hours ischemia–reperfusion-injured brain with the wet/dry method. In sham operated mice, water content in the brain was approximately 77%. On the other hand, saline-injected mice exhibited approximately 80% water content in the brain. \( Agtr^{2+} \)-MSC-injected mice showed significantly lower water content compared with that in saline-injected mice. Interestingly, treatment with \( Agtr^{2+} \)-MSC injection did not show a beneficial effect on brain edema.

Deletion of the AT1 Receptor Failed to Reduce Inflammatory Cytokine After Reperfusion

Proinflammatory cytokines such as tumor necrosis factor-\( \alpha \) are related to the development of brain edema. Next, we...
assessed inflammatory cytokines in the brain after ischemia–reperfusion injury. Tumor necrosis factor-α and monocyte chemoattractant protein-1 mRNA expression were increased in the ischemic brain. Treatment with Agtr2+ MSC suppressed the increase in tumor necrosis factor-α and monocyte chemoattractant protein-1 mRNA expression (Figure 4A–B), but not monocyte chemoattractant protein-1 expression (Figure 4B). In contrast, treatment with Agtr2− MSC did not attenuate the increase in tumor necrosis factor-α and monocyte chemoattractant protein-1 expression in the contralateral hemisphere, which was significantly increased in Agtr2− MSC-injected mice compared with that in saline-injected mice (Figure 4A).

**Increase in Methyl Methanesulfonate-Sensitive 2 Expression in Marrow Stromal Cells**

Next, we compared cell characteristics between Agtr2+ and Agtr2− marrow cells. There was no difference in morphological characteristics and proliferative activity, which was evaluated by methylcellulose-based colony-forming unit assay of colony-forming unit-macrophages and colony-forming unit-granulocytes and macrophages using whole marrow cells between them (93.8 ± 8.2 in Agtr2+ and 95.2 ± 9.9 in Agtr2− per well of a 24-well culture dish), indicating that there was no difference in the number of stem progenitor cells between Agtr2+ and Agtr2− marrow cells. Next, we analyzed MMS2 expression in marrow stromal cells from Agtr2+ MSC and Agtr2− MSC by real-time reverse transcriptase–polymerase chain reaction methods. MMS2 was significantly highly expressed in Agtr2+ MSC compared with that in Agtr2− MSC (Figure 5).

**Effect of Angiotensin II Type 1 Receptor Blocker on Survival Rate After Ischemia–Reperfusion Injury**

Finally, we assessed the effect of treatment with an AT1 receptor blocker, valsartan, on MSC. Interestingly, valsartan-treated Agtr2+ MSC-transplanted mice exhibited no operative death until 6 days after ischemia–reperfusion injury. Moreover, treatment of Agtr2+ MSC with valsartan increased the survival rate up to 80%, similar to that in the Agtr2+ MSC-transplanted group without valsartan treatment, as shown in Figure 6. These results suggest that AT1 receptor blockade and consequent AT2 receptor stimulation with unbound angiotensin II could contribute to the protective effects of MSC.

**Discussion**

Therapeutic benefits of MSC after stroke have been highlighted. Our present findings demonstrate the possibility that...
the AT$_2$ receptor is an important molecular determinant of MSC-induced cerebroprotection after stroke. Accumulating evidence from recent major clinical trials indicates that blockade of renin–angiotensin system is effective to prevent a first or recurrent stroke, independent of the blood pressure-lowering effect. The AT$_2$ receptor may be stimulated by unbound angiotensin II together with AT$_1$ receptor blockade during AT$_1$ receptor blocker treatment. The contribution of endogenous MSC to brain protection after stroke in humans is a matter of debate; however, our findings could provide a new insight that relative stimulation of AT$_2$ receptor signaling, with blockade of the AT$_1$ receptor and simultaneous AT$_2$ receptor stimulation by AT$_3$ receptor blocker treatment, could have a therapeutic advantage to prevent neurological disorders after stroke.

Although MSC have been reported to have beneficial effects after stroke such as through their plasticity or ability to secrete growth or protective factors, the detailed mechanism is totally unknown. After injection of PKH-stained MSC or MSC obtained from GFP mice through the tail vein after reperfusion, only few fluorescent cells were observed in the brain after ischemia–reperfusion injury (data not shown), indicating that only a very small amount of MSC could stay in the ischemic region. Therefore, it is difficult to consider that injected MSCs in situ could act as cellular repair factors. Accordingly, in the present study, we considered that MSC could act as an indirect neuroprotective factor to regulate the production and secretion of growth factors and/or cytokines rather than as a direct factor by cellular replacement.

In our study, monocyte chemotactrant protein-1 expression tended to be decreased in Agrt2$^{-}$-MSC but not in Agrt2$^{+}$-MSC. However, we could not have a statistical significant difference of monocyte chemotactrant protein-1 level between Agrt2$^{-}$- and Agrt2$^{+}$-MSC-injected groups. Therefore, we speculated that the marked decrease in survival rate in Agrt2$^{-}$-MSC-injected mice after ischemia–reperfusion injury was at least partly due to an increase in an inflammatory cytokine, tumor necrosis factor-$\alpha$. The anti-inflammatory effect of MSC has focused attention on them as potential therapeutic agents in disorders of the immune system to achieve effects by transplantation. Endogenous tumor necrosis factor-$\alpha$ and matrix metalloproteinase-9 were increased after MCA occlusion in the brain of rodents. Hosomi et al reported that treatment with antitumor necrosis factor-$\alpha$ neutralizing antibody reduced brain infarct volume and cerebral edema, which are likely to be mediated by a reduction in matrix metalloproteinase upregulation. Therefore, MSC injection-induced reduction of tumor necrosis factor-$\alpha$ may prevent brain edema and mortality of cerebral ischemia. Interestingly, in Agrt2$^{-}$-MSC-injected mice, tumor necrosis factor-$\alpha$ expression was induced in the contralateral side of the brain, indicating that Agrt2$^{-}$-MSC injection through the tail vein induced an inflammatory response even in the ipsilateral area as well as having less potential for brain protection than Agrt2$^{+}$-MSC injection. Moreover, in an inflammatory microenvironment, MSCs were rejected by the inflammatory response in an investigation of the plasticity and long-term survival of GFP-labeled MSC transplanted into the normal brain. Therefore, an increase in inflammation also inhibited prevention of brain damage by MSC. Further investigation of the time course of ischemic area and brain edema will clarify the mechanisms of this beneficial effect of injected MSC to induce brain protection.

Recently, we reported that AT$_3$ receptor signaling induces neurite outgrowth through transactivation of MMS2 expression involving the association of AT$_2$ receptor interacting protein and tyrosine phosphatase, SHP-1. Our recent data also demonstrated that AT$_3$ receptor signaling-induced MMS2 upregulation stimulated the DNA repair pathway and decreased DNA damage after ultraviolet radiation. After reperfusion, tissues are exposed to oxidative stress, thereby resulting in neural DNA damage. One possibility is that the loss of function in Agrt2$^{-}$-MSC is due to an increase in DNA damage and loss of number after exposure to reperfusion injury, partly due to decreased expression of MMS2. However, it is difficult to count the number of MSCs after their injection through the tail. Therefore, further investigation is necessary to prove this hypothesis.

Treatment with AT$_3$ receptor blocker, valsartan, cancelled a failure of brain protective effect in Agrt2$^{-}$-MSC-injected mice. Previously, our report demonstrated that deletion of AT$_2$ receptor increases stroke size after MCA occlusion and valsartan reduced stroke size in wild-type mice. Although the animal model for stroke was different from the previous paper, similar brain protective effect through AT$_3$ receptor stimulation was observed. In the present study, we also showed that brain-protective effects through angiotensin receptors by not only an AT$_2$ receptor stimulation, but also a blockade of AT$_3$ receptor signaling. The correlation between 2 types of angiotensin receptors in MSC has been further investigated in our laboratory.

Taking these findings together, we conclude that stimulation of AT$_3$ receptor signaling in MSC plays a pivotal role in the contribution of MSC treatment to brain protection after focal brain ischemia–reperfusion injury.

**Disclosures**

None.

**References**


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