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

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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 (Agtr₂⁻) and the effect of selective AT₁ receptor blocker.

Methods—Wild-type mice (Agtr₂⁺) were subjected to 3 hours of focal brain ischemia followed by reperfusion (ischemia–reperfusion injury). Simultaneously, Agtr₂⁺-MSC, Agtr₂⁻-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 Agtr₂⁺-MSC-injected mice, and 20% in Agtr₂⁻-MSC-injected mice. Neurological deficit after ischemia–reperfusion injury was improved in Agtr₂⁺-MSC-injected mice, but not in Agtr₂⁻-MSC-injected mice. After 48 hours of ischemia–reperfusion injury, brain infarct size was reduced in Agtr₂⁺-MSC-injected mice, but not in Agtr₂⁻-MSC-injected mice. Moreover, brain edema was significantly ameliorated in Agtr₂⁺-MSC-treated mice but not in Agtr₂⁻-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 Agtr₂⁺-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 Agtr₂⁻-MSC-treated mice. In contrast, monocyte chemoattractant protein-1 levels tended to increase Agtr₂⁻-MSC-treated mice without a significant difference. Treatment of MSC with an AT₁ receptor blocker, valsartan, significantly improved survival rates in Agtr₂⁻-MSC-injected mice.

Conclusions—These results suggest that AT₁ 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
(AT2) 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 AT1 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 (Agr2−/−; based on C57BL/6J strain) and wild-type mice (Agr2+/-; 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 (bilateral 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 10−7 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 Agr2−/− and Agr2+/- 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 were 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 (Agr2−/-MSC), angiotensin II type 2 receptor-deficient mice (Agr2−/-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 Agr2−/-MSC injection survived after ischemia–reperfusion injury. Interestingly, Agr2−/-MSC-injected mice showed a marked

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Neurological score was improved in Agtr2 mice 24 hours after MCA occlusion/reperfusion. Mice exhibited neurological deficits after MCA occlusion and stained with 2,3,5-triphenyltetrasodium chloride (Figure 2A). After 48 hours of ischemia–reperfusion injury, decrease in survival rate to approximately 20% 6 days after ischemia–reperfusion injury. There was a significant difference in survival rate by log-rank test in Agtr2 - versus Agtr2 -MSC-injected mice and saline- versus Agtr2 -MSC-injected mice. Twenty-four hours after ischemia–reperfusion injury, blood level was increased in Agtr2 -MSC transplantation compared with saline-injected mice, whereas blood level was lower in Agtr2 -MSC injected mice compared with Agtr2 -MSC transplantation mice as shown in the Supplemental Table. In contrast, blood CO2 level was lower compared with saline or Agtr2 -MSC injected mice. On the other hand, glucose levels were not changed in these mice. Mice exhibited neurological deficits after MCA occlusion such as hemiplegia, loss of balance, and no spontaneous motor activity. Neurological deficit was evaluated by neurological score 24 hours after MCA occlusion/reperfusion. Neurological score was improved in Agtr2 -MSC-treated mice compared with saline-injected mice; however, such improvement was not observed in Agtr2 -MSC-injected mice (Figure 1B). Moreover, neurological deficit was significant higher in Agtr2 -MSC-treated mice than Agtr2 -MSC-treated mice, indicating that lack of AT2 receptor signaling in MSC attenuated the effects of MSC treatment to improve survival and motor activity after focal brain ischemia.

**Treatment With Marrow Stromal Cells Did Not Induce a Significant Reduction of Ischemic Brain Area or Improvement of Cerebral Blood Flow After Ischemia–Reperfusion Injury**

Next we examined the reasons why the survival rate was attenuated in Agtr2 -MSC-injected mice. Brain samples were obtained 24 and 48 hours after ischemia–reperfusion injury and stained with 2,3,5-triphenyltetrasodium chloride (Figure 2A). In saline-injected control mice, the maximal ischemic area was found in section 4, and was approximately 25% of the total area after 48 hours of ischemia–reperfusion injury. In saline-injected control mice, the ischemic volume was approximately 50 mm3 in 24 hours and 80 mm3 in 48 hours (Figure 2B). After 48 hours of ischemia–reperfusion injury, treatment with Agtr2 -MSC showed a significant reduction of ischemic size but not with Agtr2 -MSC as shown in Figure 2B compared with that in saline-injected mice. Moreover, we measured cerebral blood flow in the core and peripheral regions of the MCA territory just before and 1, 2, and 3 hours after MCA occlusion and 1 hour and 24 hours after reperfusion (Supplemental Figure II). After MCA occlusion, cerebral blood flow in both the core and periphery decreased with an approximately 90% reduction of the basal level in the core and a 40% reduction in the periphery. After removing the nylon filament, cerebral blood flow was increased to approximately 50% of the basal level and increased up to 70% in the core and 50% in the periphery. Treatment with Agtr2 - MSC after reperfusion had a tendency to improve cerebral blood flow; however, no significant change was obtained until 24 hours after reperfusion. Furthermore, blood–brain barrier damage evaluated by Evans blue injection 24 hours after ischemia–reperfusion injury was not different in each mouse (data not shown).

**Lack of AT2 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,33 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. Agtr2 -MSC-injected mice showed significantly attenuated water content compared with that in saline-injected mice. In contrast, treatment with Agtr2 -MSC injection did not show a beneficial effect on brain edema (Figure 3).

**Deletion of the AT2 Receptor Failed to Reduce Inflammatory Cytokine After Reperfusion**

Proinflammatory cytokines such as tumor necrosis factor-α are related to the development of brain edema.34 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 ischemic area compared with the saline-injected group. Interestingly, tumor necrosis factor-α expression in the contralateral hemisphere 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...
Therefore, we speculated that the marked decrease in survival rate 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 AT1 receptor blocker, valsartan, cancelled a failure of brain protective effect in Agtr2−/−MSC-injected mice. Previously, our report demonstrated that deletion of AT2 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 AT2 receptor stimulation was observed. In the present study, we also showed that brain-protective effects through angiotensin receptors by not only an AT2 receptor stimulation, but also a blockade of AT1 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 AT1 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.

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