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; Tepepi 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 (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 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

Received December 26, 2007; accepted February 6, 2008.
From the Department of Molecular Cardiovascular Biology and Pharmacology, Ehime University, Graduate School of Medicine, Tohon, Ehime, Japan.
Correspondence to Masatsugu Horiuchi, MD, PhD, FAHA, Department of Molecular Cardiovascular Biology and Pharmacology, Ehime University, Graduate School of Medicine, Shitsukawa, Tohon, Ehime 791-0295, Japan. E-mail horiuchi@m.ehime-u.ac.jp
© 2008 American Heart Association, Inc.
Stroke is available at http://stroke.ahajournals.org
DOI: 10.1161/STROKEAHA.107.513275

2554
(AT$_2$) receptor and type 2 (AT$_2$) receptor. Although the majority of angiotensin II actions are mediated through the AT$_1$ receptor, accumulating evidence has suggested that the AT$_2$ receptor in general not only opposes the AT$_1$ receptor, but also has its own effects independent of an interaction with AT$_1$ receptor signaling. We reported that activation of the AT$_2$ 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 paramural region in mice subjected to middle cerebral artery (MCA) occlusion. Moreover, we demonstrated that AT$_2$ 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 AT$_2$ receptors may promote cell differentiation and regeneration in neuronal tissue. Li et al reported that AT$_2$ 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 AT$_2$ receptor activation and is involved in cerebellar development. This accumulating evidence indicates that AT$_2$ 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 AT$_2$ 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 AT$_2$ receptor activation in tissue regeneration. However, the roles of the AT$_2$ receptor in MSC transplantation after stroke have never been investigated. Here, we examined the possibility that stimulation of AT$_2$ 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 AT$_2$ receptor-deficient mice (Agrtr2$^{\text{-/-}}$; based on C57BL/6J strain) and wild-type mice (Agrtr2$^{\text{+/+}}$; 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 AT$_1$ receptor blocker, valsartan (provided by Novartis Pharma AG) at a dose of 10$^{-7}$ mol/L. MSC (2.0$\times$10$^5$ cells suspended in 200 $\mu$L) were injected through the tail vein after diluting in phosphate-buffered saline (200 $\mu$L) 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$\times$100.

Progenitor Colony Formation Assays

Fresh Agrtr2$^{\text{-/-}}$ and Agrtr2$^{\text{+/+}}$ 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$\times$10$^5$ cells per plate. After 2 weeks, the colonies were scored using a dissecting microscope at $\times$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 AT$_2$ 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 (Agrtr2$^{\text{-/-}}$-MSC), angiotensin II type 2 receptor-deficient mice (Agrtr2$^{\text{-/-}}$-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 Agrtr2$^{\text{-/-}}$-MSC injection survived after ischemia–reperfusion injury. Interestingly, Agrtr2$^{\text{-/-}}$-MSC-injected mice showed a marked...
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
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_1 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 chemoattractant protein-1 expression tended to be decreased in Agtr_2^-MSC but not in Agtr_2^+MSC. However, we could not have a statistical significant difference of monocyte chemoattractant protein-1 level between Agtr_2^- and Agtr_2^-MSC-injected groups. Therefore, we speculated that the marked decrease in survival rate in Agtr_2^-MSC-injected mice after ischemia–reperfusion injury was at least partly due to an increase in an inflammatory cytokine, tumor necrosis factor-α. 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-α and matrix metalloproteinase-9 were increased after MCA occlusion in the brain of rodents. Hosomi et al reported that treatment with antitumor necrosis factor-α 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-α may prevent brain edema and mortality of cerebral ischemia. Interestingly, in Agtr_2^-MSC injected, tumor necrosis factor-α expression was induced in the contralateral side of the brain, indicating that Agtr_2^-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 Agtr_2^-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_1 receptor signaling induces neurite outgrowth through transactivation of MMS2 expression involving the association of AT_1 receptor interacting protein and tyrosine phosphatase, SHP-1. Our recent data also demonstrated that AT_1 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 Agtr_2^-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_1 receptor blocker, valsartan, cancelled a failure of brain protective effect in Agtr_2^-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_1 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_1 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_1 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**

37. Cowen TM, Marcus AJ, Woodbury D, Black IB. Marrow stromal cells transplanted to the adult brain are rejected by an inflammatory response and transfer donor labels to host neurons and glia. Stem Cells. 2006;24:2483–2492.
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, Masaki Mogi, Jian-Mei Li, Kana Tsukuda, Li-Juan Min, Akiko Sakata, Teppei Fujita, Masaru Iwai and Masatsugu Horiuchi

Stroke. 2008;39:2554-2559; originally published online July 10, 2008;
doi: 10.1161/STROKEAHA.107.513275
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/39/9/2554

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
hhttp://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/