Human Dental Pulp-Derived Stem Cells Protect Against Hypoxic-Ischemic Brain Injury in Neonatal Mice

Mari Yamagata, DDS; Akihito Yamamoto, DDS, PhD; Eisuke Kako, MD, PhD; Naoko Kaneko, MD, PhD; Kohki Matsubara, DDS; Kiyoshi Sakai, DDS, PhD; Kazunobu Sawamoto, PhD; Minoru Ueda, DDS, PhD

Background and Purpose—Perinatal hypoxia-ischememia (HI) has high rates of neurological deficits and mortality. So far, no effective treatment for HI brain injury has been developed. In this study, we investigated the therapeutic effects of stem cells from human exfoliated deciduous teeth (SHED) for the treatment of neonatal HI brain injury.

Methods—Unilateral HI was induced in postnatal day 5 (P5) mice. Twenty-four hours later, SHED, human skin fibroblasts, or serum-free conditioned medium derived from these cells was injected into the injured brain. The effects of cell transplantation or conditioned medium injection on the animals’ neurological and pathophysiological recovery were evaluated.

Results—Transplanted SHED, but not fibroblasts, significantly reduced the HI-induced brain-tissue loss and improved neurological function. SHED also improved the survival of the HI mice. The engrafted SHED rarely differentiated into neural lineages; however, their transplantation inhibited the expression of proinflammatory cytokines, increased the expression of anti-inflammatory ones, and significantly reduced apoptosis. Notably, the intracerebral administration of SHED-conditioned medium also significantly improved the neurological outcome, inhibited apoptosis, and reduced tissue loss.

Conclusions—SHED transplantation into the HI-injured brain resulted in remarkable neurological and pathophysiological recovery. Our findings indicate that paracrine factors derived from SHED support a neuroprotective microenvironment in the HI brain. SHED graft and SHED-conditioned medium may provide a novel neuroprotective therapy for HI. (Stroke. 2013;44:551-554.)

Key Words: cell transplantation ■ functional recovery ■ inflammation ■ neonatal ischemia ■ stem cells ■ trophic factors

Materials and Methods

An expanded version of the Methods section is available in the online-only Data Supplement. SHED, human skin fibroblasts, and their serum-free conditioned medium (CM) were prepared as described. The SHED’s multi-differentiation potential and their expression of both mesenchymal stem cell and neural lineage markers were similar to those reported previously. HI brain injury was induced in postnatal day 5 (P5) mice as described. Cells (2×10⁵) in 2 μL phosphate buffered saline or phosphate buffered saline alone (as a control) were transplanted into the ipsilateral hemisphere at 2.0 mm anterior and 2.0 mm lateral to bregma, and 2.0 mm deep to the dural surface, using a glass needle and a Kopf microstereotaxic injection system, 24 hours after HI (Figure 1A). These animals were given daily administration of cyclosporin A (Novartis, Nurnberg, Germany, 10 mg/kg, IP) throughout the experimental period, except when they were used for cytokine expression analysis. For the experiments using CM, mice were given a 2-μL injection of CM or Dulbecco’s modification of Eagle’s medium (as a control) without cyclosporin A treatment. The animals’ neurological recovery was examined by a foot-fault test in 4-, 6-, and 8-week-old HI mice. Tissue loss was examined by staining with hematoxylin and eosin, and brain injury was evaluated using a neuropathological scoring system, by an observer blinded to the identity of the animal group. The level of apoptosis was analyzed by staining with anticaspase-3 (Cell Signaling). Real-time reverse transcription PCR was carried out as described. GAPDH cDNA was amplified as an internal control. Primer sequences are shown in the online-only Supplemental Table 1. Data are expressed as means±SEM. Survival data were analyzed by applying the Kaplan-Meier curve, followed by the Mental-Cox
log-rank test to identify differences between the curves. Behavioral
data were analyzed by 2-way ANOVA. Comparisons of parameters
among the groups were made by 1-way ANOVA. Post-hoc analyses
were performed with Bonferroni test. All statistical analyses
were performed with Stata version 11.0 (Stata Corp, College Station, TX).
A value of $P<0.05$ was considered statistically significant.

Figure 1. Neurological outcomes and survival rate after HI. A, Experimental protocol. Mice underwent HI insult at P5, and then received
cell transplantation (Cell) or CM injection (CM) at P6. In the analyses of cell transplantation’s effects on functional recovery and brain
damage, the phosphate buffered saline-, Fb-, and SHED-treated groups received daily administrations of cyclosporin A (CsA). The
cytokine assay and brain damage evaluation were performed at P7 and P8, respectively. Neurological recovery was examined at 4, 6,
and 8 weeks. B, Survival curve (SHED n =14; Fb n=12; PBS n = 18; SHED-CM n = 16; Fb-CM n = 16; DMEM n = 16; Sham n = 5).
C, Foot-fault test (SHED n=12; Fb n=9; PBS n = 9; SHED-CM n = 15; Fb-CM n = 10; DMEM n = 13; Sham n = 5). D, Statistic data.
Values are means±SEM, *$P<0.05$, **$P<0.01$.

Figure 2. Histological evaluation. A, Representative HE-stained coronal brain sections. Scale bar: 1 mm. B, Cell-transplantation. C,
Conditioned medium (CM)-injection. Tissue loss (each group, n=10–12); Caspase-3 (+) cells (each group, n=6); Pathological score (each
group, n=5–8). D, Statistic data. Values are means±SEM, n=6 per group, *$P<0.05$, **$P<0.01$. 
Results

The HI mice that underwent SHED transplantation exhibited significant neurological recovery compared with the fibroblasts- and phosphate buffered saline-treated groups (Figure 1C). The SHED-transplanted group also displayed better survival over time (Figure 1B). Histological examination revealed that the tissue loss, number of apoptotic cells, and neuropathological score in the SHED-transplanted group were significantly lower than in the other experimental groups (Figure 2A and B). Cell-type analysis showed that the apoptosis of neurons in the cortex, corpus callosum, and hippocampus and of oligodendrocytes in the corpus callosum was significantly reduced in the SHED-transplanted group (online-only Supplemental Figure 2).

The expression levels of proinflammatory cytokines interleukin-1β and tumor necrosis factor-α were upregulated in the phosphate buffered saline- and fibroblasts-transplanted groups 24 hours after HI, but those of anti-inflammatory cytokines interleukin-4 and interleukin-10 were downregulated. Notably, engrafted SHED significantly suppressed the expression of proinflammatory cytokines, whereas strongly upregulating anti-inflammatory cytokines (Figure 3).

Eight weeks after transplantation, little or no SHED had differentiated into neurons, oligodendrocytes, or astrocytes (online-only Supplemental Figure 3). Taken together, these results suggested that SHED promoted recovery after HI by paracrine mechanisms. In support of this idea, we found that mice receiving a 2-µL injection of SHED-CM in the HI-injured brain exhibited significant or better recovery in neurological function (Figure 1C), survival rate (Figure 1B), and neuropathological score (Figure 2C) than those receiving fibroblasts-CM or cell-culture medium (Dulbecco’s modification of Eagle’s medium) alone.

Discussion

Here we demonstrated that the transplantation of SHED into the HI-injured mouse brain improved the neurological outcome and survival rate. The engrafted SHED shifted the HI-induced proinflammatory state to an anti-inflammatory one and inhibited apoptosis and tissue loss. Importantly, mice receiving an injection of 2 µL SHED-CM 24 hours after HI exhibited significant recovery as assessed by both neurological and pathological examinations. These results suggest that most of the SHED-mediated therapeutic benefits were elicited by paracrine mechanisms. It was difficult to compare the level of therapeutic benefits between engrafted SHED and SHED-CM, because in the SHED experiments, the administration of cyclosporin A, which protects engrafted cells from the xenogeneic host immune response, significantly suppressed the HI-induced inflammatory response and apoptosis (online-only Supplemental Figure 4). Furthermore, cell transplantation may have an advantage in providing a prolonged delivery of paracrine factors, compared with the transient delivery by the CM treatment.

Previous reports indicate that the engraftment of various types of transplanted stem cells is a promising regenerative therapy for HI. However, for clinical use, mesenchymal stem cells must be expanded by a reliable cell-culture system that produces sufficient cell numbers to elicit clinical benefits, while also meeting safety requirements. These severe restrictions may impede the progress of regenerative therapy for HI. Our data suggest that the administration of SHED-CM provides a portion of the therapeutic benefit of SHED.
transplantation, and this finding may be useful in establishing a practical regeneration therapy for HI.

Acknowledgments
We thank the Division of Experimental Animals and Medical Research Engineering, Nagoya City University Graduate School of Medical Sciences, for housing the mice and for microscope maintenance.

Sources of Funding
This work was supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Grants-in-Aid for the Practical Application of Regenerative Medicine from the Ministry of Health, Labor and Welfare of Japan, the Funding Program for Next Generation World-Leading Researchers (Japan Society for the Promotion of Science), and a Grant for COE for Education and Research of Micro-Nano Mechatronics of the Nagoya University Global COE Program.

Disclosures
None.

References
Human Dental Pulp-Derived Stem Cells Protect Against Hypoxic-Ischemic Brain Injury in Neonatal Mice
Mari Yamagata, Akihito Yamamoto, Eisuke Kako, Naoko Kaneko, Kohki Matsubara, Kiyoshi Sakai, Kazunobu Sawamoto and Minoru Ueda

Stroke. 2013;44:551-554; originally published online December 13, 2012; doi: 10.1161/STROKEAHA.112.676759
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 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/44/2/551

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/06/18/STROKEAHA.112.676759.DC1

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:
http://www.lww.com/reprints

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

Supplemental Methods

Cell Culture and CM Preparation

Human dental pulp tissues were obtained from clinically extracted, healthy deciduous teeth from three patients. The ethics committee of Nagoya University approved our experimental protocols. The SHED were isolated and cultured, and the CM was prepared as previously described\(^1\). In brief, 80% confluent cells were washed three times with PBS followed by two washes with DMEM. The cells were then incubated in DMEM for 48 hr at 37°C in 5% CO\(_2\). The CMs were collected, and cell debris was removed by centrifugation. Fb (SF-TY, JCRB0075) were purchased from Health Science Research Resources Bank in Japan.

Flow cytometry analysis showed that the SHED expressed a set of mesenchymal stem cell (MSC) markers (i.e., CD90, CD73, and CD105), but not endothelial/hematopoietic markers (i.e., CD34, CD45, CD11b/c, and HLA-DR)\(^1\). Like human BMSCs, SHED exhibited adipogenic, chondrogenic, and osteogenic differentiation as described previously\(^2,3\).

HI Induction

All animal-related procedures were approved by the Laboratory Animal Care and Use Committee of Nagoya City University and were conducted in accordance with the guidelines of the NIH. Wild-type ICR mice were purchased from SLC (Shizuoka, Japan). HI brain injury was induced in postnatal day 5 (P5) mice as described\(^4\). The pups were anesthetized with 2% isoflurane in a mixture of N\(_2\)O and O\(_2\) (2:1), and the right common carotid artery was cauterized. After a 30-minute recovery period, the pups were placed in a plastic chamber containing a humidified atmosphere of 8% O\(_2\) and 92% N\(_2\), and submerged in a 37.5°C water bath to maintain normothermia. After 20 minutes of hypoxia, the pups were returned to their dams.

Cell Transplantation and CM Injection

For cell transplantation, the cells were infused into the ipsilateral hemisphere at 2.0 mm anterior and 2.0 mm lateral to bregma, and 2.0 mm deep to the dural surface, using a glass needle and a Kopf microstereotaxic injection system. Approximately 2\(\times\)10\(^5\) cells
in 2 µl PBS were injected. The animals receiving cell transplantation were given daily injections of cyclosporin A (Novartis, Nurnberg, Germany, 10 mg/kg, i.p.) throughout the experimental period, except when the animals were used for cytokine expression analysis. For CM injection, SHED-CM, Fb-CM, or DMEM was injected into the damaged brain 24 hours after HI at the position described for cell transplantation. The CM-injected mice were maintained without cyclosporine A.

**Histology and Immunohistochemistry**
Brains were perfusion-fixed with 4% paraformaldehyde and postfixed in the same fixative overnight. Using a Vibratome sectioning system (VT1200S; Leica), 60-µm coronal sections between the corpus callosum and the dorsal hippocampus, approximately 60 sections in total, were prepared. For immunohistochemistry, every 6th section was stained with one or more of the following primary antibodies: anti-cleaved caspase-3 (Cell Signaling), anti-neuronal nuclei (NeuN; Millipore), anti-glial fibrillary acidic protein (GFAP; Sigma), anti-adenomatous polyposis coli (APC; Millipore), anti-human nuclei (HuN; Millipore), anti-microtubule-associated protein 2 (MAP2; Millipore), and anti-oligodendrocyte transcription factor 2 (Olig2; IBL). Primary antibodies were visualized with Alexa Fluor-conjugated secondary antibodies (Invitrogen). Confocal tissue images were obtained with a confocal laser microscope (LSM5 PASCAL, Zeiss), while blight field images were taken with BZ9000 (Keyence). Various brain areas were outlined manually using ImageJ software.

**Brain Damage Assessment and Apoptosis Quantification**
Tissue loss and infarct area were examined by staining with hematoxylin and eosin (HE). The percent volume loss in the ipsilateral versus contralateral hemisphere was determined for each animal as previously described. Brain injury was evaluated using a neuropathological scoring system as described previously, by an observer blinded to the animal group. The level of apoptosis was analyzed by staining with anti-caspase-3 alone or together with anti-GFAP, NeuN, or Olig2. The apoptotic cell density was calculated from the total caspase-3-positive cell numbers in a counted area.

**Neurological Evaluation**
To evaluate neurological motor function, a foot-fault test was performed in 4-, 6-, and
8-week-old HI mice, as described previously. Mice were placed on a hexagonal grid (45×55 cm, 20-cm above the floor). During locomotion, the number of foot faults made by the ipsilateral and contralateral limbs was counted. Each test consisted of 2 trials lasting 5 minutes; trials were performed on consecutive days.

RNA Isolation and RT-PCR
Total RNA isolation, RT reactions, and quantitative PCR were carried out as described. As an internal control, GAPDH cDNA was amplified. Primer sequences are shown in Supplemental Table 1.

Statistical Analyses
Data are expressed as means ± SEM. Survival data were expressed by applying the Kaplan-Meier curve, followed by the Mental-Cox log-rank test to identify differences between the curves. Behavioral data were analyzed by two-way ANOVA. Comparisons of parameters among the groups were made by one-way ANOVA. Post-hoc analyses were performed with Bonferroni’s test. All statistical analyses were performed with Stata version 11.0 (Stata Corp., College Station, TX, USA). A value of P< 0.05 was considered statistically significant.
### Supplemental table S1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Used for quantitative Real Time-PCR Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Primer sequence (5′-3′)</td>
</tr>
<tr>
<td></td>
<td>Position</td>
</tr>
<tr>
<td></td>
<td>Size of Product (bp)</td>
</tr>
<tr>
<td>forward</td>
<td>AAATTTGGCATTGAGGAAGGT</td>
</tr>
<tr>
<td>reverse</td>
<td>GGATGCAAGGATGTGTTCT</td>
</tr>
<tr>
<td>forward</td>
<td>CTTCTACTCTGACCCCTTATTGT</td>
</tr>
<tr>
<td>reverse</td>
<td>TGCCCAGCATCCTGTTGTTCT</td>
</tr>
<tr>
<td>forward</td>
<td>CCCTTTACTCTGACCCCTTATTGT</td>
</tr>
<tr>
<td>reverse</td>
<td>GCCGATGATCCTGCTCAAGTATTG</td>
</tr>
<tr>
<td>forward</td>
<td>GTGCTCAACCCCCAGCTAGT</td>
</tr>
<tr>
<td>reverse</td>
<td>GCCGATGATCCTGCTCAAGTATTG</td>
</tr>
<tr>
<td>forward</td>
<td>GGCTTCAGCCAAGCTAGT</td>
</tr>
<tr>
<td>reverse</td>
<td>GCCGATGATCCTGCTCAAGTATTG</td>
</tr>
<tr>
<td>forward</td>
<td>CCAAGAAGGATGCTCAATTACAGA</td>
</tr>
<tr>
<td>reverse</td>
<td>CATCGTCCCCAGAAGGCAAC</td>
</tr>
<tr>
<td>forward</td>
<td>GGCCTGACTGAGCACATGAG</td>
</tr>
<tr>
<td>reverse</td>
<td>CGAAGCTAGGAAGCATGAG</td>
</tr>
<tr>
<td>forward</td>
<td>CCTGACTCTTCTGCTGCTTTT</td>
</tr>
<tr>
<td>reverse</td>
<td>GGCCTTCTGATGATGCTCATA</td>
</tr>
<tr>
<td>forward</td>
<td>TCTCCCAGAAGGACTCTTAC</td>
</tr>
<tr>
<td>reverse</td>
<td>AACTGGTTCCTCTGATGACAGA</td>
</tr>
</tbody>
</table>
Supplemental figure S2

A

PBS-treated HI injured brain

SHED-treated HI injured brain

GFAP

NeuN

APC

B

Caspase3+ cells/mm²

Cortex

CC

Striatum

Hippocampus

GFAP

NeuN

APC

others
Supplemental figure S3
Supplemental figure S4
Supplemental figure legends

Figure S2. SHED transplantation inhibits HI-induced apoptosis.
(A) Representative images stained with anti-caspase-3 together with GFAP, NeuN, or APC. (B) Characterization of the types of apoptotic cells. SHED transplantation significantly reduced the apoptosis of neurons in the striatum, hippocampus and cortex, and of oligodendrocytes in the CC. Values are means ± SEM per mm3, n=5 per group, *P < 0.05, **P < 0.01, ***P < 0.001. Scale bar: 20 µm.

Figure S3. Engrafted SHED had rarely differentiated into neurons, oligodendrocytes, or astrocytes.
Immunohistochemical analysis with an anti–human nuclei monoclonal antibody (HuN) together with antibodies against GFAP (A), MAP2 (B), or Olig2 (C). Scale bar: 50 µm.

Figure S4. The effect of cyclosporine A treatment on the cytokine expression.
Cyclosporine A treatment significantly decreased the expression of pro-inflammatory IL-1β, but up-regulated the anti-inflammatory IL-6 and IL-10. Values are means ± SEM. *P < 0.05.
Supplemental references


