The Simultaneous Treatment of MMP-2 Stimulants in Retinal Transplantation Enhances Grafted Cell Migration into the Host Retina

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ABSTRACT
The success of functional retinal cell transplantation has been limited by the low efficiency of the transplanted cell integration into the host retina. Given that the extracellular matrix (ECM) is thought to inhibit entry and axonal outgrowth of grafted neural cells into the host retina, modulation of the ECMS in the host environment may overcome this limitation. Here, we demonstrate that matrix metalloprotease-2 (MMP-2) expression is associated with the high migratory potential of adult rat hippocampus-derived neural stem cells compared with retinal progenitor cells. In addition, MMP-2, as well as its reported inducers concanavalin A and 17β-estradiol, can trigger the migration of retinal progenitor cells into explanted retinas. Inhibitors of MMP-2 suppressed these effects. Intense cell migration is not required for photoreceptor transplantation; however, the environment that allows the transplanted cells to integrate is most important. Migration of the transplanted cells is a good index of the acceptance of grafted cell of the host tissue. Strategies modulating the environment by MMP-2 stimulation may provide an advance in the development of retinal transplantation.

INTRODUCTION
Neural cell transplantation is a promising therapeutic option to replace dysfunctional cells in degenerative diseases of the central nervous system (CNS), including retina. Retinal transplantation, however, has achieved only limited success as yet [1–9]. Successful retinal transplantation requires incorporation of the graft cells, proper synapse formation between host and grafted neural cells, and long-term survival of the functional grafted cells.

The primary obstacle in retinal transplantation has been poor incorporation of the grafted cells into the retina, the initial step in transplantation. Kinouchi et al. recently demonstrated that elimination of the intermediate filaments of reactive Müller cells or astrocytes within the retina permitted neuronal integration in retinal transplantation of glial fibrillary acidic protein (GFAP)/−/−;vimentin−/− mice [9]. This result suggested that removal of the extracellular matrix (ECM) barrier produced by reactive glial cells in the damaged host retina is important in encouraging the acceleration of graft cell migration.

A member of the matrix metalloproteinases (MMPs), a family of zinc-dependent proteases that can degrade components of the ECM, is a potential candidate for this purpose [10, 11]. MMP expression is associated with tumor invasion and metastatic potential [10–12]. MMPs also contribute to oligodendrocyte neurite outgrowth in the CNS or axonal growth in peripheral nerves by inhibition of factors, such as chondroitin sulfate proteoglycan, and modulation of the ECM. In previous studies, we observed the migration and incorporation of adult rat hippocampus-derived neural stem cells (AHSCs) (clone PZ5) into the developing retina and into the damaged retinas of adult rats [13–16]. Although incorporated AHSCs mimic retinal cells morphologically, they do not express retinal cell markers after settlement [17–19]. In contrast, retinal progenitor cells express retinal markers after transplantation, but the efficiency of grafted cell incorporation into the host retina is too low for practical use in transplantation [20, 21].

In this study, we showed for the first time that MMP-2 might be responsible for the invasive nature of the grafted progenitor cells and that the manipulation of host environment with MMP-2 or its stimulator facilitate the migration of retinal progenitor cells into explanted retinas. Although the photoreceptors exist in the outermost layer of the retina, the transplanted pho-
toreceptor cells should be incorporated with host retina and extend their processes into it. To evaluate this ability of integration, we adapted the migration ratio of the transplanted cells as an index.

**MATERIALS AND METHODS**

**Animals**

All experimental procedures in this study adhered to the Guidelines for Animal Experimentation of Kyoto University (Kyoto, Japan). Animals were maintained in a constant environment on a 14-hour light-dark cycle. Animals were provided with water and food ad libitum.

**Reverse Transcription-Polymerase Chain Reaction**

Total RNA was isolated from both embryonic day 19 (E19) retina of Fisher rats and cultured AHSCs using the guanidine isothiocyanate-based TRIzol reagent (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). First-strand cDNA was synthesized using a First-Strand CDNA Synthesis Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK, http://www.gehealthcare.com) in accordance with the manufacturer’s protocol. The polymerase chain reaction (PCR) was performed with Advantage 2 Taq polymerase (Clontech, Mountain View, CA, http://www.clontech.com) and the following primers:

- MMP-2: TGCACCATGCCATCATCAAGTT (sense), AAGGCCCAGCAAAGCATCATCC (antisense);
- MMP-7: TTGAGGACTCCAGACATCATAAT (sense), GCTGAGAAGGCCGGTTGTGTCCAT (antisense);
- MMP-9: GCCCAACAGCTCCTCCCACTATG (sense), TTGCGGCCAGAGAGAAGAATAC (antisense);
- RX: GCTACTCGCCCTGCTATCC (sense), AGGGCGGTGGCGGCGTGTA (antisense); and
- PAX6: CCGGGAAAGACTAGCAGCCAAAAT (sense), AAGGCCCGAGCAAAAGCATCATCC (antisense).

In the PCRs, an initial denaturation at 94°C (1 minute) was followed by amplification with 28 cycles of denaturation at 94°C (30 seconds), annealing at 58°C (30 seconds), and extension at 68°C (1 minute). For each primer pair, a positive control (ovine thyroid gland) and a negative control (water) were included in each PCR.

**Coculture of Retinal Progenitor Cells with Retinal Explant**

Retinal explant cultures were performed as described [22, 23]. Briefly, eyes of adult Fisher rats were enucleated (Shimizu Laboratory Supplies, Kyoto, Japan, http://web.kyoto-inet.or.jp/people/simizu/index.htm); each neural retina was removed from the remaining tissue and placed on a Millicell-CM chamber filter (30-mm diameter, 0.4-μm culture plate insert; Millipore, Billerica, MA, http://www.millipore.com) with the ganglion cell inhibitor group), 10^−10 M E_2 and MMP-2 inhibitor (ex vivo E_2/MMP-2 inhibitor group), 20 μg/ml Con A and 10^−10 M E_2 (ex vivo Con A/E_2 group), 20 μg/ml Con A, 10^−10 M E_2, MMP-2 inhibitor (ex vivo Con A/E_2/MMP-2 inhibitor group), 20 μg/ml MMP-2 inhibitor (ex vivo MMP-2 inhibitor group), or no additional substances (control group). For ex vivo transplantation, retinal cells from P0-P2 green fluorescent protein (GFP) transgenic mice (the kind gift of M. Okabe, Osaka University, Osaka, Japan) were dissociated using the Papain Dissociation System (Worthington Biochemical Corporation, Lakewood, NJ, http://www.worthington-biochem.com). Two microliters of cell suspension (1.0 × 10^5 cells per microliter) were placed between the host retina and the chamber filter (subretinal space). Explants were cultured at 34°C in 5% CO_2, with a change of media every other day; samples were harvested and processed for histology approximately 1 week later.

**Tissue Processing**

To prepare retinal sections, animals were sacrificed with an overdose of pentobarbital sodium 4 weeks after surgery. Animals were perfused transcardially first with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany, http://www.merck.com) in 0.1 M phosphate buffer. The posterior segments of the enucleated eyes were immersed in fresh 4% PFA at 4°C for 16 hours, then in 25% sucrose-PBS for cryoprotection. After being embedded in an optimal cutting temperature compound (Bayer Corp., Emeryville, CA, http://www.bayer.com), consecutive 12–16-μm frozen sections were generated on a cryostat.

**Immunohistochemistry**

After washing in PBS, sections were preincubated in blocking solution (PBS containing 20% skim milk and 0.3% Triton X-100) for 30 minutes, then incubated overnight at 4°C with one of the following antibodies diluted as specified in 5% skim milk and 0.3% Triton X-100 in PBS (staining buffer): mouse or rabbit anti-GFP antibody (1:500; Molecular Probes Inc., Eugene, OR, http://probes.invitrogen.com). Subsequent immunofluorescence staining used goat anti-mouse immunoglobulin G (IgG) (H+L) (AlexaFluor 488; Molecular Probes Inc.) or goat anti-rabbit IgG (H+L) (AlexaFluor 594; Molecular Probes Inc.), as appropriate, each diluted 1:500 in the staining buffer. GFP was visualized either directly or indirectly by staining using mouse or rabbit anti-GFP (1:500; Molecular Probes Inc.).
TRANSPLANTATION. In the ex vivo MMP-2 group, 18.43% MMP-2 stimulates cell migration into the host retina in ex vivo SCs (green) into the retina completely. This result indicates that MMP-2 functions in the migration of AHSCs (Fig. 1).

To investigate whether these effects were mediated by MMP-2, we added an MMP-2 inhibitor into these ex vivo transplantation, the explants were examined by immunohistochemistry using an anti-β-gal (green). Note the extensive migration of AHSCs (green) into the retina (B) in comparison with that seen in (C). Scale bars = 20 μm. Abbreviations: AHSC, adult rat hippocampus-derived neural stem cell; E19, embryonic day 19; GCL, ganglion cell layer; INL, inner nuclear layer; MMP-2, matrix metalloprotease-2; ONL, outer nuclear layer; SUB, subretina.

RESULTS

The Function of MMP-2 Expression by AHSCs in Cell Migration

Of all of the MMPs examined by preliminary reverse transcription-PCR analysis, only MMP-2 was upregulated in AHSCs in comparison with the levels observed in embryonic retinal cells (E19) (Fig. 1). Neither MMP-7 nor MMP-9 mRNA was expressed in both cell types (data not shown).

To investigate the role of MMP-2 in the migratory capacity of AHSCs during retinal transplantation, we examined whether exogenous MMP-2 inhibitors affected the migration of AHSCs into the host retina using retinal organ culture. Seven days after ex vivo transplantation, AHSCs migrated well into the entirety of the host retina. In contrast, addition of an MMP-2 inhibitor abrogated AHSCs migration into the host retina nearly completely. This result indicates that MMP-2 functions in the migration of AHSCs (Fig. 1).

Effects of MMP-2 on Retinal Progenitor Cell Migration in Retinal Explants

Next, we used dissociated retinal cells derived from newborn GFP+ mice as donor cells to investigate whether exogenous MMP-2 stimulates cell migration into the host retina in ex vivo transplantation. In the ex vivo MMP-2 group, 18.43% ± 1.60% of the total GFP+ cells migrated into host retina (Fig. 2), a significantly greater proportion than observed in the ex vivo control group (2.46% ± 0.54%, p < .01) (Fig. 2). We then examined the effect of Con A, E2, and the combination of both stimuli on cell migratory potential. These substances enhance MMP-2 activity by protein activation and transcriptional up-regulation, respectively. The application of Con A, E2, or both increased the percentages of migrating cells (15.68% ± 2.86%, 12.88% ± 2.29%, and 17.72% ± 2.43%, respectively; Figs. 2 and 3). These increases were statistically significant (p < .05, ex vivo Con A group vs. ex vivo control group; p < .01, ex vivo E2 group vs. ex vivo control group). Although there was no additive effect on the percentage of the migrated cells when the Con A and E2 treatments were given together, the cells increasingly migrated into the inner retina after combined treatment (Fig. 3D; Table 1).

To investigate whether these effects were mediated by MMP-2, we added an MMP-2 inhibitor into these ex vivo transplantation conditions (Fig. 4; Table 1). When an MMP-2 inhibitor was added, the ratio of migrated cells in each condition decreased (7.54% ± 0.55%, 5.82% ± 1.06%, and 11.71% ± 1.66% for the ex vivo Con A/MMP-2 inhibitor, ex vivo E2/MMP-2 inhibitor, and ex vivo Con A/E2/MMP-2 inhibitor groups, respectively).

![Figure 1](image1.png)

**Figure 1.** The secretion of MMP-2 from AHSCs is important in the migratory plasticity of retinal and/or retinal progenitor cells transplanted into a host retina. (A): Reverse transcription-polymerase chain reaction (RT-PCR) analysis of retinal cells and AHSCs. Total RNA was extracted from both E19 retinas of C57Bl/6 mice and cultured AHSCs cells, then analyzed by RT-PCR. (B, C): Sections of retinal explants untreated (B) or treated with an MMP-2 inhibitor (C). One week after cell transplantation, the explants were examined by immunohistochemistry.

![Figure 2](image2.png)

**Figure 2.** The effect of exogenous MMP-2 on the migratory plasticity of retinal and/or retinal progenitor cells. (A, B): Sections of retinal explants in the absence (A) or presence (B) of active MMP-2. One week after cell transplantation, explants were subjected to immunohistochemistry labeled with anti-green fluorescent protein antibody (green). The transplanted cells in the MMP-2 group (B) exhibited improved migration in comparison with the control group (A). Scale bars = 20 μm. (C): The number of transplanted cells in each group which migrated to the host retina were quantified 1 week after transplantation. In comparison with the control group, the MMP-2 group exhibited a significant increase in the ratio of transplanted cells that migrated into the host retina. **p < .01 versus control. Error bars indicate mean ± SD. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; MMP-2, matrix metalloprotease-2; ONL, outer nuclear layer; SUB, subretina.**
DISCUSSION
To achieve the functional recovery of a damaged CNS by cell transplantation, the donor transplant cells must migrate and integrate into the neural circuit at the damaged site. Grafted stem cells appear to have the ability to migrate toward damaged sites, taking their cue from a chemoattractant produced at the site [24, 25]. Degradation of the ECM appears to be necessary for the grafted cells to integrate into a host tissue packed with cells. We observed that the migration ratio (percentage of transplanted cells entering the retina) differs significantly among the various types of neural stem/progenitor cells. Prestoz et al. also observed this phenomenon, reporting that differences in integrin signaling contributed to the differential migratory abilities [26]. Thus, the migration of transplanted cells are significantly influenced by the ECM. The robust neuronal integration in retinal transplantation seen in GFAP−/−/vimentin−/− mice also supports this viewpoint [9].

MMPs are proteolytic enzymes that degrade ECM molecules, including collagen, fibronectin, laminin, and a variety of proteoglycans. These proteases have been widely implicated in tumor invasion and metastasis [10–12]. Recently, the role of MMPs in the generation of the CNS and peripheral nervous system has been increasingly recognized. MMP-2 and MMP-9 expression increases at sites of peripheral nerve injury or degeneration and enhances neural promoting activity, partially by downregulating the chondroitin sulfate proteoglycan activity that may inhibit neural outgrowth [12, 13]. In the CNS, MMP-9 contributes to process outgrowth by oligodendrocytes in the initial phase of myelination that occurs during development or during remyelination after pathologic damage [14, 15]. The efficient migratory potential and axonal outgrowth of AHSCs hinted to us that factors degrading the ECM may facilitate the invasive nature of these cells. We found that the expression of MMP-2 is higher in AHSCs than in embryonic retinal progenitor cells. The migratory potential of AHSCs could be abrogated by treatment with an MMP2 inhibitor, indicating an important role for MMP-2 in the migration of AHSCs during retinal transplantation. In ex vivo transplantation, exogenous activation of MMP-2 enhanced the migration of retinal progenitor cells into the host retina, suggesting that the activation of MMP-2 in situ may trigger migration and enhance the incorporation of grafted cells into the host retina.

MMP-2 activity can be regulated at three levels: gene transcription, proenzyme activation by membrane-type metalloproteases (MT-MMP), and inhibition by naturally occurring tissue inhibitors of metalloproteinases (TIMPs) [27]. Phorbol esters, interleukin-1, tumor necrosis factor-α, transforming growth factor β-1, and E2 are all stimulants of MMP-2 gene transcription. Sp1, Sp3, AP-2, and Smad4 are transcription factors reported to upregulate MMP-2 mRNA levels [28–32]. MMP-2 is initially expressed as an inactive proenzyme, which is

Table 1. A semiquantitative analysis of the transplanted cell quantity in the different layers

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Abbreviations: Con A, concanavalin A; E2, 17β-estradiol; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; MMP-2, matrix metalloprotease-2; ONL, outer nuclear layer; OPL, outer plexiform layer; SUB, subretina; ±, one or two cells per field; +, several cells per field; ++, more than 10 cells per field; −, no cells per field.
activated by MT-MMP cleavage of the propeptide, rendering the enzyme fully active. TIMPs form a complex with both pro-MMP-2 and active MMP-2, which controls the rate of MMP-2 activation in a complicated manner [27].

The plant lectin Con A, a carbohydrate-binding protein, induces MT1-MMP expression, subsequently activating MMP-2 [33]. Pretreatment of myoblasts with Con A increased the intramuscular migration of transplanted myoblasts in an MMP-2-dependent manner [34, 35]. Con A was also implicated as a neuronal modulator, as the extracellular domains of many receptors for neurotransmitters, growth factors, or other neuro-modulators are glycosylated, leading to ligation by lectins such as Con A [36, 37]. E2 regulates the expression of MMP-2 by activating estrogen receptors in a dose-dependent fashion in both in vivo and in vitro systems [31, 32]. In the CNS, E2 induces synaptogenesis in the hippocampus and dendritic spine formation in hippocampal neurons in a brain-derived neurotrophic factor-dependent pathway [38, 39].

In this study, the migratory activity of transplanted cells was enhanced by adding Con A, E2, or the combination to the culture medium; these effects could be significantly suppressed by the addition of an MMP-2 inhibitor. This result suggested that Con A and E2 may facilitate grafted cell migration by activating MMP-2 in situ. The fact that we did not observe any additive effects on the percentage of migrating cells after treatment with both stimuli indicates that both of these factors affect the same pathway. Interestingly, combined treatment resulted in a significantly different distribution of the migrated cells within the retinal explant, indicating the potential existence of an additive effect of these two factors on a pathway independent from MMP-2. Simultaneous treatment of retinal progenitor cells with Con A and E2 also enhanced the migration of grafted cells into the retinas of mice exhibiting retinal degeneration. This is the first report conclusively demonstrating that stimulation of MMP-2 activity can enhance the incorporation of grafted cells during neuronal transplantation.

The recovery of retinal function depends primarily on the efficiency of grafted cell integration into the host retina. Intense cell migration is not required for photoreceptor transplantation; however, the environment that allows the transplanted cells to integrate is most important. Migration of the transplanted cells is a good index of the acceptance of grafted cells of the host tissue. Strategies modulating the environment by MMP-2 stimulation may provide an advance in the development of retinal transplantation therapy.

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DISCLOSURES
The authors indicate no potential conflicts of interest.

REFERENCES


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