p27 modulates tropism of mesenchymal stem cells toward brain tumors

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Abstract. Mesenchymal stem cells (MSCs) have inherent tumor-tropic properties in the brain and seem to be a useful tool for cellular therapy for brain tumors. However, the mechanisms involved in MSC migration are not fully understood. The tumor suppressor p27, an inhibitor of cyclin-dependent kinase complexes, not only plays a crucial role in cell cycle regulation but also has cell cycle-independent functions, such as differentiation and migration of cells. In fact, p27 has been alternatively reported to inhibit or stimulate cell migration in cells of different types. Therefore, in the present study, we investigated whether p27 is involved in the tumor-tropic activity of MSCs using MSCs from p27-null mice. It was found that $p27^{-/-}$ MSCs showed a decreased motility in the wound healing assay and displayed increased numbers of stress fibers. To compare the *in vivo* migratory activity of $p27^{-/-}$ and $p27^{+/+}$ MSCs toward glioma, we injected C6 glioma cells into one side of the mouse brain and BrdU-labeled $p27^{-/-}$ or $p27^{+/+}$ MSCs into the other side. Significantly fewer labeled $p27^{-/-}$ MSCs were observed in the tumor area compared with $p27^{+/+}$ MSCs. The present study suggests that p27 works as a stimulator of the *in vitro* and *in vivo* migration process of MSCs toward tumors. These findings are important when the efficacy of stem cell-based strategies for glioma therapy is considered.

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Introduction

Glioblastomas, the most common primary malignant brain tumors, infiltrate the surrounding normal brain tissues, and therefore are almost always non-curable even with surgical resection. Despite recent refined therapeutic strategies, the regrowth of tumor cells residing in the adjacent brain inevitably occurs, resulting in a dismal prognosis for patients with glioblastoma (1). Recently, mesenchymal stem cells (MSCs) that have inherent tumor-tropic properties have been tested as a vehicle for delivery of therapeutic genes such as suicide genes and cytokine genes in experimental gliomas (2-13). MSCs used as a vehicle for suicide gene therapy have obtained sufficiently effective results both in intracranial glioma (14) and in the leptomeningeal glioma models (15). However, little is known about the mechanisms involved in the migratory activity of MSCs toward tumors.

Cyclin-dependent kinase (CDK) inhibitor $p27^{Kip1}$ is a well-characterized tumor suppressor and is frequently down-regulated by enhanced degradation of p27 in malignancies. The decreased expression of p27 is usually correlated with increased tumor aggressiveness and poor clinical outcome. Notably, high p27 levels correlate with high tumor grade, poor prognosis and increased metastasis. This has been observed, for instance, in various types of tumors (breast, cervix, esophagus and uterus) and in certain types of lymphomas and leukemias (16-18). These observations suggest that deregulation of p27 in tumors may serve to uncouple it from its cell cycle-inhibitory function, possibly by being excluded from the nucleus. Once in the cytoplasm, p27 may exert other functions, such as the regulation of cell migration, thereby promoting tumor progression and invasiveness (19,20).

When MSCs are used as a vehicle for glioma gene therapy, highly migratory MSCs would be more efficient. However, little is known about whether p27 is involved in the tumor-tropic properties of MSCs. In the present study, we investigated the influence of p27 on MSC migration by using MSCs derived from p27-null and wild-type mice and found that the motility of $p27^{-/-}$ MSCs was impaired and the numbers of actin stress fibers of these cells were increased. The *in vivo* migratory

activity of the $p27^{-/-}$ MSCs toward the tumor in the mouse brain was lower than that of the $p27^{+/+}$ MSCs, suggesting that p27 acted as a stimulator during the migration process of MSCs.

Materials and methods

Isolation and culture of MSCs. All following experiments were performed according to the Rules of Animal Experimentation and the Guide for the Care and Use of Laboratory Animals of the Hamamatsu University School of Medicine. $p27^{-/-}$ and $p27^{+/+}$ C57BL/6 mice (8 weeks old) were sacrificed with ether, and the marrow tissue was obtained from the femurs and tibias as previously described (21). A single-cell suspension was obtained by gently aspirating the tissue several times using the same needle and syringe in 5 ml Murine MSC Growth Medium (MMSCGM; StemCell Technologies Inc., British Columbia, Canada), washed one time with 10 ml fresh MMSCGM and passed through a 70-μm nylon strainer (Falcon, Becton Dickinson Labware, Franklin lakes, NJ, USA). The cells were then plated into a 25-cm^2 tissue culture flask in 5 ml MMSCGM and incubated at 37˚C under 5% CO_2. The non-adherent cells were removed by replacing the medium 24 h after the initial culture. The residual attached cells were maintained at 37˚C in 5% CO_2 by exchanging the medium with fresh medium at 5-day intervals. These cells are designated as MSCs in the present study.

Wound healing assay. Cells were seeded at 80% confluence in 60-mm dishes and grown for an additional 24 h. A linear scratch, ~1 cm wide, was performed using a rubber policeman across the diameter of the plate. This was then rinsed with phosphate-buffered saline (PBS). Cells were fed with growth medium supplement. Cells were incubated for 24 h, rinsed with PBS, and fixed for 5 min in 95% ethanol/5% acetic acid at room temperature. For each plate, images were captured using a dissection microscope (Zeiss) at a magnification of x20. Then the distance the cells had migrated from the scratch line at each time point was measured in mm. Cells were pretreated with 10 μg/ml mitomycin C for 3 h to block cell division in order to rule out the possibility that the differences in motility were due to the differences in cell proliferation.

BrdU labeling and immunohistological analysis. For the *in vivo* transplantation experiments, MSC cultures grown for 5 days were pulsed for 48 h with 5 μM 5-bromo-2-deoxyuridine (BrdU) (Sigma) in Eagle's minimal essential medium supplemented with 10% FBS or for 24 h with 10 μg/ml bisbenzimide (Sigma) before harvest (22). Cells were harvested by incubation with 0.25% trypsin for 5 min at room temperature followed by gentle scraping. For BrdU immunostaining, the DNA was first denatured by incubating the brain sections (6 μm) in 50% formamide 2X SSC at 65˚C for 2 h and then in 2 N HCl at 37˚C for 30 min. The sections were then rinsed with PBS and treated with 1% H_2O_2 to block endogenous peroxidase. The sections were incubated with a mouse monoclonal antibody agaist BrdU (1:500, Sigma) overnight and incubated with biotinylated secondary antibody (Dako) for 1 h. Control experiments consisted of staining brain coronal tissue sections as described above, but the primary antibodies were omitted.

In vivo migratory capacity of MSCs toward brain tumors in nude mice. To compare the *in vivo* migratory capacity and tropism of $p27^{-/-}$ and $p27^{+/+}$ MSCs toward glioma, we injected $2x10^4$ C6 rat glioma cells into one side of the mouse brain hemisphere and $1x10^5$ BrdU-labeled MSCs into the opposite hemisphere. The method of cell implantation was the same as described previously (21). Briefly, 10 BALB/c nude mice (6 weeks old, Nippon SLC, Hamamatsu, Japan) were anesthetized with 0.4 ml/100 g equithesin and placed in a stereotaxic apparatus (Narishige Scientific Instrument Lab., Tokyo, Japan). A 25-gauge needle was inserted into the target point (0.2 mm posterior to the bregma, 2 mm left of the midline, 3 mm ventral from the dura), and $2x10^4$ C6 cells were injected with a 10-μl microsyringe (Hamilton Company, Reno, NV, USA) and a microinjector (Harvard Apparatus Inc., South Natick, MA, USA) for 5 min. After one week, $2x10^4$ BrdU-labeled MSCs ($p27^{-/-}$ or $p27^{+/+}$, n=5 for each group) were injected at the mirror point in the contralateral hemisphere (0.2 mm posterior, 2 mm right). The animals were sacrificed on day 10. Serial coronal sections (5 μm) were obtained and stained with hematoxylin and eosin, and the tumor area of each section was measured using NIH Image software (rsbweb.nih.gov). The adjacent sections were stained with the BrdU antibody as described above, and the MSC infiltrating area was detected. The migration potential of MSCs was defined as the MSC infiltration area/C6 tumor region.

Results

$p27^{-/-}$ MSCs exhibit no different morphological characteristics but have elevated growth. The murine $p27^{Kip1}$ genomic locus comprises three exons spanning ~4 kb. The targeting construct was designed to delete the exon 1 and 2 of the $p27^{Kip1}$ gene, since the protein-coding region resides only in exons 1 and 2 (23). MSCs were isolated from $p27^{-/-}$ and $p27^{+/+}$ mice as described in Materials and methods. There was no difference in the morphological characteristics between $p27^{-/-}$ and $p27^{+/+}$ MSCs, but the $p27^{-/-}$ MSCs had an elevated proliferation rate compared to $p27^{+/+}$ MSCs due to the function of p27 as an inhibitor of CDK. The doubling growth time of $p27^{+/+}$ MSCs (122 h) was more than twice that of $p27^{-/-}$ MSCs (60 h).

Impaired motility in $p27^{-/-}$ MSCs. To further characterize the function of p27 during MSC migration, the motility of MSCs derived from $p27^{-/-}$ and $p27^{+/+}$ mice was measured using the wound healing assay. The wound healing assay was designed as a method of simulating the ability of a cell to reconstruct a tissue. Cell motility in this assay system is dependent upon reorganization of the actin cytoskeleton and the assembly and disassembly of focal adhesion complexes, processes which are governed by the Rho family GTPases Rho, Rac and Cdc42 (24). Following wounding of a confluent cell monolayer, $p27^{-/-}$ MSCs exhibited a significant reduction in cell motility compared with cells derived from wild-type MSCs 24 h later ($p<0.05$, Fig. 1).

Increased numbers of actin stress fibers in $p27^{-/-}$ MSCs. It was reported that $p27^{-/-}$ fibroblasts have elevated amounts of endogenous Rho-GTP due to inhibition of RhoA by p27. $p27^{-/-}$ fibroblasts had Rho-dependent cellular phenotypes, including

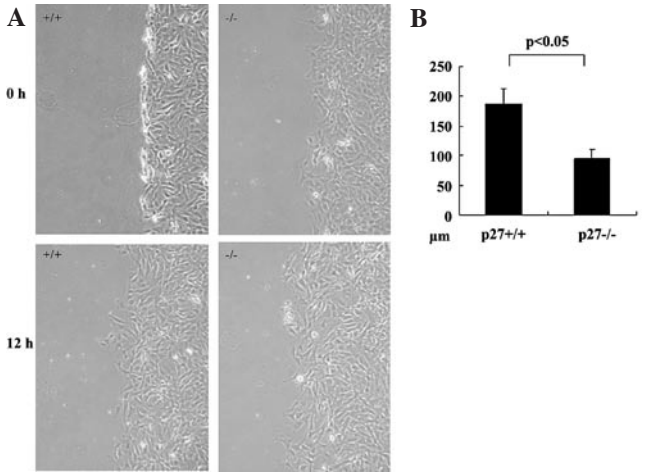

Figure 1. p27$^{-/-}$ MSCs exhibit a migration deficit in the wound healing assay. (A) Migration of p27$^{+/+}$ and p27$^{-/-}$ MSCs following wounding of a confluent cell monolayer, x20. (B) The distance migrated from the scratch line by the cells is significantly shorter in p27$^{-/-}$ MSCs than in p27$^{+/+}$ MSCs (triplicate, mean ± standard error, p<0.05).

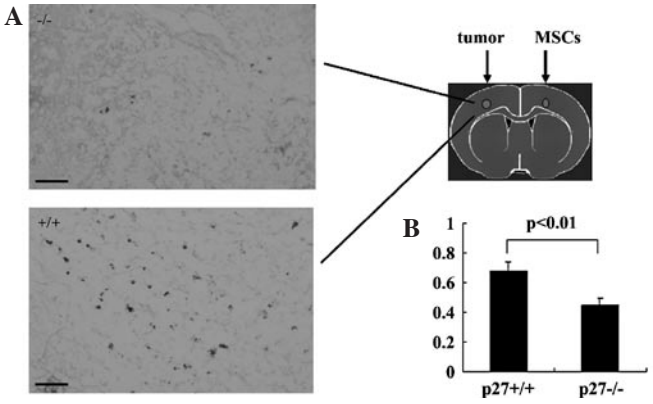

Figure 3. p27$^{-/-}$ MSCs exhibit a migration deficit toward the tumor region *in vivo*. (A) BrdU-labeled MSCs migrating from the opposite hemisphere entered the tumor region as detected using the BrdU antibody (brown-stained cells). (B) The ratio of the area of the BrdU-labeled MSC infiltration region divided by the area of the tumor region is significantly lower in p27$^{-/-}$ MSCs compared with p27$^{+/+}$ MSCs (n=5 for each group, mean ± standard error, p<0.01).

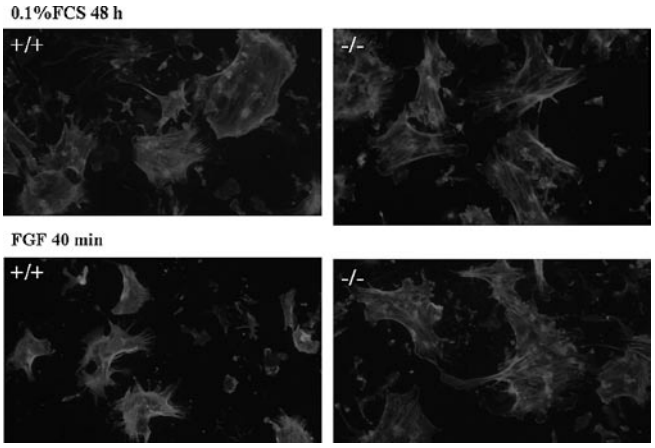

Figure 2. p27$^{-/-}$ MSCs have increased numbers of actin stress fibers. (Top) MSCs were seeded on glass coverslips and allowed to grow for 16 h, then starved for 48 h in 0.1% BSA. (Bottom) After starvation, MSCs were stimulated for 40 min with 25 ng/ml FGF. Actin was visualized with phalloidin-rhodamine (1/500).

increased numbers of focal adhesions and actin stress fibers, increased phosphorylation of cofilin (a target molecule of the Rho pathway), and a marked decrease in motility (25). We therefore surveyed the actin cytoskeleton of p27$^{-/-}$ and p27$^{+/+}$ MSCs by immunocytochemistry using phalloidin. Since various cytokines (including EPO, IL-6, SDF1-β, FGF and VEGF) showed the ability to increase the migratory activity of MSCs, we analyzed the effects of FGF on the actin cytoskeleton. Wild-type MSCs had few actin stress fibers in serum-starved conditions, and FGF stimulation evoked a dramatic rearrangement of the actin cytoskeleton (Fig. 2, left). In contrast, p27$^{-/-}$ cells had an extensive network of stress fibers in the absence of serum, and FGF stimulation substantially failed to induce the actin rearrangement (Fig. 2, right).

Decreased in vivo migratory capacity of p27$^{-/-}$ MSCs toward tumors. MSCs have the capacity to migrate specifically toward tumors. To further compare the tropism of p27$^{-/-}$ and p27$^{+/+}$ MSCs, we detected their migratory capacity toward tumors. We injected BrdU-labeled MSCs on the opposite hemisphere of the tumor inoculation. MSCs migrated across the corpus callosum toward the tumor and ultimately entered the tumor on the opposite side of the brain (Fig. 3A). We compared the tropism and infiltrative potential of p27$^{+/+}$ and p27$^{-/-}$ MSCs using the ratio of the area of the BrdU-labeled MSC infiltration region divided by the area of the tumor region. We found significantly fewer labeled p27$^{-/-}$ MSCs in the tumor compared with p27$^{+/+}$ MSCs (p<0.01, Fig. 3B). Very few p27$^{+/+}$ MSCs were found in the normal brain tissue beyond the injection site.

Discussion

Since Aboody and colleagues (26) first demonstrated the potent migratory ability of neural stem cells to brain tumors, this ability of neural stem cells has been confirmed by numerous studies, including ours (27). It has also been demonstrated that an established rat glioma can be successfully treated by genetically engineered neural stem cells (21). However, there is a limitation to using neural stem cells obtained from the patients with glioblastomas due to the invasiveness and the low proliferative activity of neural stem cells obtained from adult brains. As an alternative, we have been testing the use of MSCs obtained from the bone marrow of patients instead of neural stem cells. It has previously been demonstrated that rat brain tumors can be effectively treated by rat MSCs transduced with the herpes simplex virus-thymidine kinase gene, the same gene we used with neural stem cells (14). If MSCs can be used for the treatment of malignant glioma that deeply infiltrates the surrounding normal brain tissues, the potency of the tumor-homing activity of MSCs is particularly important for the success of this treatment strategy. However, the precise mechanisms of tumor tropism of neural and other types of stem cells are still unknown. Some recent reports suggest several molecular mechanisms of MSC migration

toward gliomas (28-30). p27, a CDK inhibitor, has also been known to regulate cell migration as well as cell proliferation. Therefore, in the present study we investigated the role of p27 on the migratory activity of MSCs.

We measured the motility of MSCs derived from p27-null mouse using a wound healing assay and found that $p27^{-/-}$ MSCs had decreased motility and increased numbers of actin stress fibers. Consistent with the results in mouse embryonic fibroblasts, p27 works as a stimulator during the migratory process of MSCs via modulation of the Rho pathway (25). MSCs are known to have strong tropism toward tumors, and when MSCs are implanted in one hemisphere and tumor cells in the other hemisphere, most MSCs travel across the corpus callosum and gather around the tumor (5,8,9,12,13,31). Our *in vivo* study, using a contralateral hemisphere injection model, demonstrated that the migratory activity of MSCs toward tumors was also significantly decreased in $p27^{-/-}$ MSCs.

p27 generally suppresses CDK activity in proliferating cells. Another role of p27 in cell migration has been recently suggested *in vitro*. However, the physiological importance of p27 in cell migration remains elusive, since p27-deficient mice have no obvious migration defect-related phenotypic abnormality. p27 has been alternatively reported as an inhibitor or stimulator of cell migration in primary or stabilized cells of different origins. Controversial results for the role of p27 in cell motility are mainly attributable to both types of cells and the methods of motility assay used. Cell migration is a dynamic process governed by intracellular and extracellular stimuli that promote the formation of focal adhesions between the cell membrane and the extracellular matrix. In some cell types p27 increases cell motility, whereas in others it decreases it. These differences possibly originate from cell type-specific variation in the relative balance between Rho and Rac activity.

Motility assays mainly consist of the fibronectin-coated transwell assay and the wound healing assay. Cell migration through the pores of fibronectin-coated transwells is promoted by amoeboid movement that responds to attractant stimuli lacking an obvious polarization, which depends largely on propulsive forces and on cytoplasmic streaming for their motility (32). In this case, p27 may act as an inhibitor of cell migration by altering microtubule stability, which then impairs the cytoskeletal modifications necessary for the cell to move. The wound healing assay is an *in vitro* directional motility assay designed to simulate the ability of cells to reconstruct tissues. It is possible that p27, contributing to stabilize the perinuclear network of microtubules, enforces cell polarity and favors the movements of highly polarized cells. $p27^{-/-}$ fibroblasts failed to reorient glutamylated microtubules to the scratched area during the wound healing assay, since they migrated toward the wounded area with altered cell trajectories (33). Accordingly, p27 expression stimulates wound healing cell motility by decreasing the RhoA-ROCK1 activity and inhibits the Rho pathway by blocking the guanine-nucleotide exchange-mediated activation of Rho (25).

Our data suggested that p27 increases cell migration under both *in vitro* and *in vivo* conditions as shown previously (25). It seems that p27 regulates the migration process of MSCs via modulation of the Rho pathway, since $p27^{-/-}$ MSCs showed increased numbers of stress fibers and were largely refractory to FGF stimuli. p27 may be acting as a tumor suppressor and as an oncogene, depending on its subcellular localization, which may explain the different regulatory functions of p27 in different cell lines. The results of the present study would open a window on the mechanisms contributing to the regulation of MSCs migration, though further studies are obviously required to understand the behaviors of stem cells in the brain. We believe that knowledge of the mechanisms involved in the migratory process and enhancement of the potency of MSC migration are directly related to the efficacy of stem cell-based strategies for glioma therapy.

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