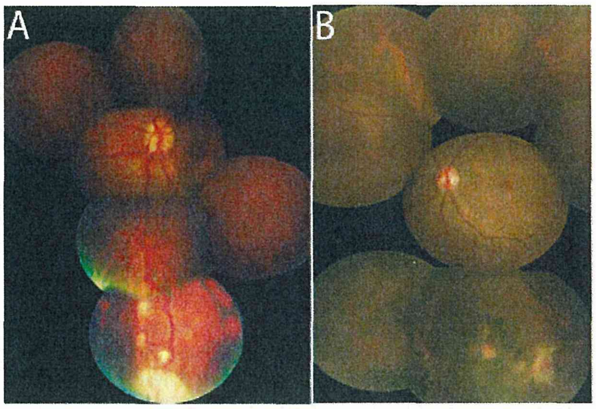

Figure 4 Fundus photograph before and after laser photocoagulation in a patient with VHL disease.

A 21-year-old female shows retinal capillary hemangioma at the inferior periphery (A) before treatments. She eventually has received laser photocoagulation 21 times at outpatient ward. At the age of 44, fundus delimits the tumor lesion with regression of the feeding vessels (B).

hemangiomas could be destroyed by single brachytherapy. They also concluded that a favorable outcome could be expected if a hemangioma's diameter is 5.0 mm or smaller and if there is no preoperative exudative retinal detachment [42]. External beam radiotherapy has been also shown to be useful when standard therapy has not prevented progression [43]. Palmer et al. have found proton-beam irradiation to be an efficacious and safe treatment for large retinal hemangiomas, measuring more than 3 mm, and for cases complicated by exudative retinal detachments or for tumors involving the optic nerve [44]. However, there may exist a problem if the tumor recurs. In such cases, it may be hard to conduct additional radiotherapy to the eye. Combination therapies including ruthenium plaque radiotherapy, cryotherapy, and PDT can be required to induce complete tumor regression and sclerosis of the dilated vessels [18] in selected cases.

Anti-VEGF agents are also candidates for retinal hemangiomas in VHL patients. There are two ways for administration of anti-VEGF agents to the human body: systemically and intravitreously. Intravitreal injections of anti-VEGF therapy (pegaptanib) may decrease retinal thickening minimally and reduce retinal hard exudates in some patients with advanced hemangiomas in patients with VHL [45]. However, the efficacy of agents in this class such as VEGF receptor inhibitor SU5416, and anti-VEGF agents including bevacizumab, ranibizumab and pegaptanib are uncertain [46-50].

Interferon (IFN)-α has an established role in cancer therapy in some cancer types such as hairy cell leukemia and melanoma. Niemela et al. reported that recombinant human IFN-α-2a (Roceron-A; Roche) was injected subcutaneously into VHL patients at a dose of 3×10^6 IU, 3 times/week for 12 months. There was a transient decrease in size and fluorescein leakage from the retinal hemangioma during the therapy. They concluded that IFN-α-2a might decrease blood flow in hemangiomas as suggested by shrinkage and diminished leakage of retinal hemangiomas [51].

On the other hand, larger tumors may have been shown to be non-responsive to medical treatments. A retrospective study of patients showed that for lesions between 7–9 mm, surgical resection of retinal tumors improved visual acuity or kept it the same [35,52]. Therefore, surgical resection of the tumor should be considered for patients with large retinal hemangiomas. The surgery should consist of pars plana vitrectomy, argon endolasing of the feeder vessels, endodiathermy of the vascular lesion, artificial posterior vitreous detachment formation, and filling of the vitreous cavity with silicone oil [35]. Expected intraoperative or postoperative complications include cataract, hemorrhage during resection, epiretinal membrane, and intraoperative retinal breaks, and recurrent retinal detachments [52]. Bimanual technique is useful to reduce intraoperative bleeding and to resect tumor tissues safely during vitrectomy [27].

Managements of optic disc/juxtapapillary hemangioma

Ophthalmologists may choose observation unless the associated visual impairments happen in patients with optic disc/juxtapapillary hemangioma, because overtreatments may lead to an irreversible optic nerve disorder. Instead, the treatments should be considered if the optic disc tumors complicate serous retinal detachment and retinal exudation formation in the macula, and subsequent visual disturbance. Laser photocoagulation may be applied if the optic disc is completely covered with the tumor, which should be confirmed using FA. Even though the tumor partially involves the optic disc, laser may be possible in case of cooperative patients to the treatments, and favorable vision fixation during laser irradiation. Otherwise, the laser photocoagulation should be avoided.

PDT can be effective in reducing macular edema associated with retinal hemangioma; however, this does not always correspond with an improvement in visual acuities especially for juxtapapillary tumors, which is more characteristic for VHL-positive patients [53]. Reynolds et al. reported that VHL patients with juxtapapillary hemangioma could experience treatment complications, including a vitreous hemorrhage and rhegmatogeneous retinal detachment. At that time, scleral buckling procedure, vitreoretinal surgery, and endo-laser photocoagulation may be required [54].

Anti-VEGF agents are also candidate treatments for optic disc hemangioma. von Below et al. demonstrated that bevacizumab, a humanized anti-VEGF antibody, was also used systemically (6mg/kg body weight); treatment decreased tumor exudation transiently, but did not improve eventual visual outcome [50]. Aiello et al. reported the treatment involving the systemic administration of a VEGF receptor inhibitor SU5416. The juxtapapillary hemangioma did not result in a decrease in tumor size but effected an improvement in visual acuity and visual field [46]. As mentioned above in the peripheral retinal hemangioma, effects of anti-VEGF treatments on suppression of tumor growth vary in each case [55]. The reasons may be related to a reduction in vasopermeability, because there was no apparent effect of treatment on the size of the primary retinal hemangiomas [45].

Matsuo et al. reported an 18-year-old woman with optic disc hemangioma in the background of VHL disease [56]. The patient underwent low-dose external beam radiation (20 Gy) to the eye using a lens-sparing single lateral technique, which led to the inhibition of visual disturbance associated with serous retinal detachment. Therefore, the authors recommended low-dose external beam radiation as the initial treatment option for optic disc hemangioma [56]. In contrast to such destructive therapies, infrared diode laser transpupillary thermotherapy provides a useful modality in the treatment of retinal capillary hemangiomas, and may be particularly favorable for juxtapapillary lesions because of its relatively nondestructive characteristics [57] in selected cases.

Corticosteroids have a significant anti-angiostatic capacity. The primary mechanism of action of angiostatic steroids appears to be in aiding breakdown and blockage of the formation of capillary endothelial basement membranes [58]. Toyokawa et al. reported a case of juxtapapillary hemangioma successfully treated with intravitreal injection of bevacizumab combined with posterior subtenon injection of triamcinolone acetonide (TA) (1.25 mg bevacizumab and 20 mg TA) [59]. Suh et al. showed that verteporfin PDT combined with intravitreal TA appeared to cause involution of the hemangioma with reduction in macular edema and improvement in visual acuity [60]. Therefore, it is likely that TA should be considered one of therapeutic options for patients with retinal and juxtapapillary hemangiomas.

Future prospects on therapeutic approach

Propanolol is a β-blocker commonly used in cardiology that may induce endothelium vasoconstriction and inhibit endothelial proliferation. It has been shown to be effective in infantile facial hemangiomas, and proved safe and effective for the choroidal hemangioma [61]. However, β-blocker has yet to be challenged for patients with retinal capillary hemangioma. Although β-blocker affects systemic circulation of human body including blood pressure, it may be initially tried especially for patients showing refractory to other treatments, after approving the ethical issues in the future.

Recently, adenovirus-mediated VHL intraocular gene transfer has been attempted, and VHL expression in adenovirus-mediated VHL-transduced cells was confirmed at the transcript and protein levels. Adenovirus expressing VHL led to a significant reduction in VEGF expression *in vitro* under normoxic or hypoxic conditions. Akiyama et al. demonstrated that adenovirus-mediated VHL effectively inhibited pathological angiogenesis in the monkey retina [62]. More recently, Sufan et al. analyzed adenovirus-mediated delivery of the bioengineered VHL protein, which contributed to the dramatic inhibition of angiogenesis and growth regression of human renal cell carcinoma xenografts in a dorsal skin-fold window chamber model [63]. Therefore, targeted VHL gene and protein transfer into the eye may open a novel therapeutic approach for retinal hemangioma of VHL disease in the future.

CONCLUSIONS

Recent advances in basic and clinical studies on VHL gene/protein and VHL disease have provided novel concepts in molecular pathology and clinical managements. pVHL plays a critical role in the regulation of HIF-dependent as well as HIF-independent signaling pathways. These mechanisms should underlie the pathogenesis of VHL-related retinal vascular tumors. It is still controversial whether the histological term "hemangioma" vs "hemangioblastoma" should be appropriate in calling retinal vascular tumors of VHL disease. Further morphological and histochemical analyses will be required to resolve the issue. Recent clinical studies have proved application of various therapeutic options depending on the location of retinal tumors between peripheral and optic disc/juxtapapillary hemangioma during a couple of decades. Indeed, long-term follow-up observation can be achieved in VHL disease patients, showing effectiveness of conventional standard treatments. However, there still exists a population suffering from irreversible severe visual disturbance even though the conventional treatments had been performed enough. Further challenging of molecular targeting therapy as well as development of vitreoretinal surgeries and gene/protein transfer technique may contribute to preservation of the patients' vision in the future.

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Review Article

Role of the von Hippel-Lindau Tumor Suppressor Protein in Neuronal Differentiation of Somatic Stem Cells and its Application to Neuronal Regeneration: A Review

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Keywords
- von Hippel-Lindau tumor suppressor protein
- Somatic stem cells
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- Peptide

Abstract

von Hippel-Lindau tumor suppressor (VHL) protein functions to cause somatic stem cells to differentiate into neurons. Not only VHL protein but also a peptide derived from it shows this capability of eliciting neuronal differentiation by somatic stem cells. Up to now, rodent neural stem cells (NSCs) and human hair follicle stem cells, both of which are derived from ectoderm, have been shown to undergo neuronal differentiation triggered by VHL protein or a peptide derived from it. In addition, rodent skin-derived precursors and rodent bone marrow mesenchymal stem cells, both of which are derived from mesenchyme, also can differentiate into neurons by the same method. A 15-amino-acid peptide derived from VHL protein corresponds to the part of the sequence of VHL that binds to elongin C, which sequence is considered to be a domain for neuronal differentiation. The mechanism of neuronal differentiation of somatic stem cells by VHL is suggested to be inhibition of Stat 3. When VHL protein or oligopeptide derived from it was transferred into somatic stem cells and these cells were transplanted into the central nervous system of animals modeling a neuronal disease, the implanted cells differentiated into neuronal cells, resulting in recovery of neuronal functions. These facts suggest that somatic stem cells with transferred VHL protein or oligopeptide derived from it are candidates of donor cells for regeneration therapy of intractable neuronal diseases.

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INTRODUCTION

von Hippel-Lindau disease is a hereditary multi-cancer syndrome that encompasses central nervous system hemangioblastoma, retinal angioma, renal cell cancer, and pheochromocytoma [1]. The causative gene composed of 3 exons and encoding 213 amino-acids, has been identified at chromosome 3p25 region [2]. The functions of this gene include inhibition of mRNA elongation [3], inhibition of hypoxia inducible factor (HIF)-1α [4], and induction of neuronal differentiation of neural progenitor cells [5]. These functions are normally controlled under normoxia, but not under hypoxia [4]. VHL protein is expressed in the cytoplasm of not only neural progenitor cells [5] but also in that of adult neurons [6]. The VHL protein is widely expressed in normal human tissues, but the cellular distribution of the protein is confined to the cytoplasm of specific cell types. High-level expression of the protein is observed in neural tissue, especially in Purkinje cells, Golgi type II cells, and cells in the dentate nucleus of the cerebellum, pontine nuclei, and inferior olivary nucleus of the medulla oblongata [6]. However, the significance of the expression of VHL protein in neurons has remained unclear. In addition, strong expression of VHL protein is observed particularly in the cytoplasm of renal tubule cells [6]. Since VHL protein plays an important role during neuronal differentiation [5], neuronal differentiation of somatic stem cells elicited by VHL protein might be useful for neuronal regeneration therapy by transplantation of somatic stem cells transfected with the *VHL* gene [5,7]. Here, in the context of translational research, we review the literature showing that VHL protein or a peptide derived from it promotes the differentiation of somatic stem cells into neuron-like cells [5,7], which ability suggests that these transfected stem cells might be useful as donor cells for neuronal regeneration therapy [8-12]. For use of somatic stem cells as donor cells for neuronal regenerative therapy, it is desirable that these cells differentiate into neuronal cells in the implant site. If naïve somatic stem cells are implanted into the central nervous system (CNS), they rarely survive; and those that do scarcely differentiate into neurons.

Somatic stem cells capable of neuronal differentiation are classified into ectoderm-derived stem cells and mesenchyme/mesoderm- derived stem cells. Endoderm-derived stem cells scarcely show neuronal differentiation. Up to now, somatic stem cells reported to differentiate to neuronal cells when transfected with the VHL gene or a peptide derived from VHL protein are the following: rodent neuronal progenitor cells/neural stem cells (NPSs/NSCs) [6,9], human hair follicle stem cells (FSCs) [13], rodent skin-derived precursors (SKPs) [10,12], and rodent bone marrow mesenchymal stem cells (MSCs) [11]. The methods for isolation and culturing of these cells have been previously described [8,10,13,14].

Expression of VHL protein in neuronal progenitor cells

When neuronal progenitor cells (NPCs) were obtained from the forebrain of the E12 rat fetus, the expression levels of VHL protein and microtubule-associated protein (MAP)-2 were positively correlated during the development of the neuronal progenitor cells [6]. At day 1 after NPCs were placed in primary culture with bFGF, VHL protein and neuron-specific marker microtubule-associated protein (MAP)-2 were expressed at a low level, but by day 14 high levels of them were detected. On the other hand, when NPCs were cultured with EGF instead of bFGF, these cells expressed neither protein at a high level [6].

Neuronal differentiation of neuronal progenitor cells/neural stem cells transfected with the VHL gene

Through transfection of NSCs/NPCs with a VHL protein-expressing herpes virus vector or adenovirus vector, these cells differentiated into neuron-like cells *in vitro* and *in vivo* [5,7]. It was first reported that NPCs differentiated into neuronal marker-expressing cells through transfection with the VHL protein-expressing herpes simplex virus vector [5]. Significant expression levels of VHL protein and MAP-2 were observed at 3 and 24 hours, respectively, after transfection with the *VHL* gene; and the expression of both was observed significantly earlier than that in the control (P <0.0001) [6]. In addition, through transfection with a VHL protein-expressing adenovirus vector, NPCs differentiated into neuronal marker-positive cells [7]. On the other hand, the expression levels of astrocytic marker glial fibrillary acidic protein (GFAP) and oligodendroglial marker O4 decreased in NPCs following transfection with the*VHL* gene [7]. MAP-2 and tyrosine hydroxylase (TH) increased in expression in response to the transfection. The ratio of MAP-2 positive NPCs to total NPCs was significantly much higher in VHL gene-transfected NPCs treated with glial cell line-derived neurotrophic factor (GDNF) than in control NPCs without this factor (P<0.01) [7]. In line with these findings, it was also shown that a VHL mRNA antisense oligonucleotide inhibited the differentiation of NPCs [6].

Neuronal differentiation of somatic stem cells by intracellular delivery of the VHL peptide having the sequence of the elongin C-binding site

Through intracellular delivery of the 15 amino-acid peptide comprising the binding site for elongin C within the VHL protein (VHL peptide), the induction of neuronal differentiation of NPCs was examined. Elongin C, a ligand of the VHL protein, forms the elongin BC complex with elongin B, which complex then binds to elongin A, a transcription factor that elongates mRNA, or to BC-box proteins, which inhibit signal transcription factors such as Stat3, a transcription factor related to astrocytic differentiation [15]. Therefore, a peptide containing the binding site for elongin C was speculated to have the capability to induce neuronal differentiation owing to its neuronal differentiation-inducing domain [8]. For the intracellular delivery of the VHL peptide, diloeoyl phosphatidyl ethanolamine lipid reagent for encapsulating molecules (BioPorter) was used. The BioPorter-peptide complexes become attached to the negatively charged cell surface and fuse with it, resulting in delivery of the captured protein into the cells. Four hours after the intracellular delivery, neurite outgrowth from NPCs was observed when the VHL peptide was used at a 0.3-μM concentration or above. Immunocytochemically, NPCs showed increased expression of

MAP-2 after the delivery of the peptide, but decreased expression of GFAP [8]. In the electrophysiological study, voltage-gated inward and outward currents were recorded in the whole-cell patch-clamp configuration. In whole-cell recordings of the VHL peptide-containing NPCs, the depolarizing voltage steps elicit both large outward potassium currents and fast inward Na+ currents, which are hallmark features of differentiated neurons [8]. Furthermore, through the intracellular transfer of the protein transduction domain (PTD) linked to the VHL peptide (PTD-VHL peptide), NSCs differentiated into neuronal marker-positive neuron-like cells *in vitro* [9]. The majority of NSCs with transferred PTD-VHL peptide expressed neuron-specific markers such as neurofilament (NF)-H, MAP2, and Tuj-1, whereas the minority were positive for the stem cell marker nestin or the astrocytic marker GFAP. By western blotting for NSCs done 7 days after treatment with PTD-VHL peptide, a significantly greater amount of NF-H protein was observed in the PTD-VHL-peptide-bearing cells than in the PTD-peptide alone-treated group. In addition, a significantly greater amount of MAP2-protein was seen in the former than in the latter [9].

Similarly, the transfer of PTD-VHL peptide induced neuronal differentiation in skin-derived precursors (SKPs) [10,12]. Morphological analysis of SKPs by use of phase-contrast microscopy revealed that after the treatment of SKPs with PTD-VHL peptide these cells showed significant neurite outgrowth by 24 hours after the start of treatment, whereas a peptide from another part of the sequence of the VHL protein or the PTD-alone peptide did not elicit such outgrowth. Immunocytochemical analysis of NFM in SKPs 7 days after treatment with PTD-VHL-peptide indicated significantly higher expression of these neurofilaments in PTD-VHL peptide-treated cells than in the control ones [10] (Figure 1). Reverse transcription polymerase chain reaction (RT-PCR) analysis revealed up-regulation of basic helix-loop-helix (bHLH) determination factors (neurogenin3; Ngn3) and that of bHLH differentiation factor (NeuroD) as well as down-regulation of inhibitory bHLH (Hes1) in PTD-VHL peptide-treated SKPs compared with their respective levels in the control cells [10].

In a study on human hair follicle stem cells (FSCs), neuronal differentiation of the stem cells was examined after the intracellular delivery of PTD-VHL peptide [13]. Two days after delivery of 1-μM peptide, observation by phase-contrast microscopy showed that most cells extended neurite-like cellular processes; whereas the control cells receiving just the PTD peptide elaborated significantly fewer of these processes. In this same immunocytochemical *in vitro* study, the PTD-VHL peptide-treated FSCs highly expressed various neuronal markers (MAP2, NFM, NFH, and Tuj-1); whereas those treated with PTD peptide alone showed only low expression of these markers [13].

In addition, neuronal differentiation of rat bone marrow mesenchymal stem cells (MSCs) by intracellular delivery of PTD-VHL peptide was examined *in vitro* [11]. One week after the intracellular delivery of the peptide, MSCs changed their morphology into multiple process-bearing neuron-like cells and expressed neuronal lineage markers, Tuj-1 and MAP-2. PTD-VHL peptide-bearing MSCs showed significantly higher rates of expression of both Tuj-1 and NFH than the control peptide-treated ones. Quantitative RT-PCR evaluation indicated that the MSCs with transferred PTD-VHL peptide expressed significantly more NFH mRNA or MAP2 mRNA than the ones treated with the control peptide. Western blot analysis indicated that the PTD-VHL peptide-bearing MSCs distinctly expressed NFH and MAP2 proteins, whereas the cells treated with the PTD peptide alone scarcely did so [11].

Transplantation of somatic stem cells transfected with VHL gene and that of PTD-VHL peptide-treated ones into the rodent brain

Yamada et al. transplanted somatic stem cells into the rodent brain to examine whether these cells could differentiate into functional neuronal cells and repair the damage caused by

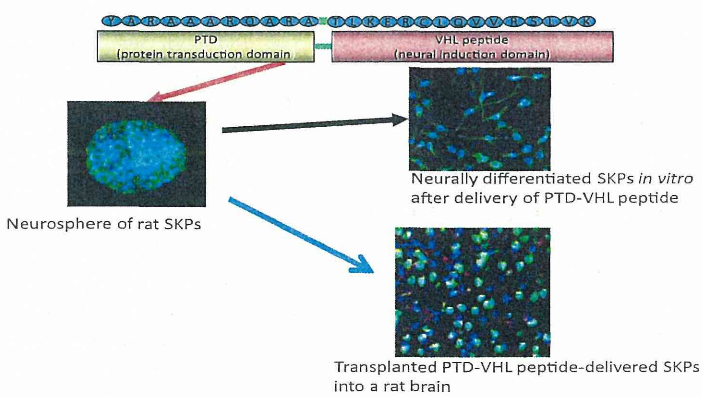

Figure 1 Intracelluar delivery of PTD-VHL peptide into peptide-delivered skin-derived precursors (SKPs) causes the cells to differentiate dinto neuron-like cells *in vitro* and *in vivo*. NA neurosphere of SKP cells with some being s showing nestin positive (green) is shown. *In vitro* neurally differentiated cells from SKPs are positive for showing NFM positive (green). Transplanted PTD-VHL peptide-bearingdelivered SKPs transplanted into a rat brain are showing NeuN positive. The transplanted cells had been were pre-labelled to emit with red fluorescence. Cellular nuclei were stained with DAPI (blue) in all figures.

neuronal diseases [7]. First of all, after NPCs had been transfected with VHL protein-expressing adenovirus vectors, the cells were transplanted into the striatum of rats modeling Parkinson's disease (PD). Prior to the transplantation, these cells were labeled with bromodeoxyuridine (BrdU). The results revealed that numerous BrdU-tyrosine hydroxylase (TH) double-labeled cells could be seen close to the transplant site, showing that the transplanted cells had efficiently generated new dopaminergic neurons within the host striatum. Moreover, all of the animals transfected with NPCs with the *VHL* gene showed a remarkable decrease in apomorphine-induced rotations. These findings thus indicate that NPCs transfected with the *VHL* gene could efficiently differentiate into dopaminergic neurons [7].

In other studies exploring the possibility that SKPs could survive and function *in vivo*, SKPs treated with PTD-VHL peptide were transplanted into the striatum of PD model rats [10,12]. The transplanted SKPs differentiated into not only TH- or dopamine transporter (DAT)-positive cells in the rat brain, but also secreted dopamine and repaired symptoms (e.g., apomorphine-induced rotations) in this PD model (unilateral 6-OHDA-lesioned rats) [12]. Two weeks after the 6-OHDA lesioning, the rats were depleted of dopaminergic innervations in their ipsilateral striatum and exhibit a characteristic rotation behavior as a response to the apomorphine challenge [12]. Functional analysis of this apomorphine-induced rotation behavior revealed that rats in the PTD-VHL-peptide-treated SKP group showed a slight but significantly greater improvement at 4 weeks after the transplantation than the rats in the non-treated SKP group or sham-operation group. To evaluate the survival and morphological maturation of SKPs grafted in the striatum of PD model rats, the cells were prelabeled with red fluorescence before grafting. The animals were then sacrified for histological analysis 8 weeks after the engraftment. No tumor formation by the grafts was found in these animals [10]. Immunohistochemical analysis of sections by use of confocal microscopy revealed that more than 5% of the fluorescence-labeled transplanted cells survived in the PD rats. In the PTD-VHL-peptide-SKP group, fluorescence -labeled cells showed high positive rate for NeuN as well as for TH; and the sprouting of dopaminergic neurons surrounding the fluorescence-labeled cells was enhanced; whereas in the sections prepared from the naive SKP group, the fluorescence-labeled cells showed a low positive rate for NeuN as well as for TH [10] (Figure 1).

PTD-VHL peptide-bearing FSCs and control ones were prelabelled with red fluorescence and separately implanted into rodent brains [13]. Three weeks later, after perfusion/ fixation, the brains were frozen with liquid nitrogen and sectioned. The number of surviving implanted cells (red fluorescence -prelabelled cells) among the implanted PTD-VHL-peptide-treated FSCs was significantly greater than that of the cells treated with PTD alone. In addition, prelabelled cells expressing Tuj-1 represented 38.8% ± 3.5% of the PTD-VHL peptide-treated cell population, which percentage was significantly greater ($p < 0.01$) than the 9.3% ± 1.5% found for the one treated with PTD peptide alone [13].

Transplantation of somatic stem cells into the spinal cord

Immunohisto chemical results obtained by confocal analysis four weeks after transplantation of NSCs into striatum revealed that the number of surviving (red fluorescence-prelabeled cells) PTD-VHL-peptide-treated NSCs was significantly greater than that of the non-treated cells ($P<0.05$). In addition, the PTD-VHL-peptide-treated fluorescence-prelabeled NSCs co-localized more with neuronal markers than with a glial marker suggesting thatdthese NSCs had differentiated into neurons rather than into astrocytes in the injured spinal cord [9].

Similarly, four weeks after transplantation of MSCs into the rat spinal cord, confocal analysis of MSCs treated with PTD-VHL peptide or PTD peptide alone showed that the number of the surviving cells (red fluorescence-prelabeled cells) was significantly greater for the former than for the latters ($P<0.01$). The data also indicated that more PTD-VHL peptide-MSCs had differentiatedeinto NFH-positive cells than the cells treated with PTD peptide alons [11].

Correlation of functional recovery in PD-model rats with dopamine levels in the lesioned striatum

The behavior of a novel population of VHL gene-transfected NPCs compared with that of non-transfected NPCs was investigated after their implantation into the adult rat striatum previously lesioned by 6-OHDA [7]. Most non-transfected transplanted NPCs differentiated into astrocytes, and only a few of them differentiated into neurons. In contrast, *VHL* gene-transfected-NPCs differentiated into neurons, with more TH-positive cells formed *in vivo* than *in vitro*. The physiological function of TH-positive cells generated from these latter NPCs was assessed in this study by analysis of recovery from apomorphine-induced rotation behavior in a rat model of PD. All of the animals transplanted with *VHL* gene-transfected-NPCs showed a remarkable decrease in apomorphine-induced rotations for up to 16 weeks. In contrast, no behavioral improvements were noted in rats after sham surgery or after transplantation with non-transfected NPCs. Therefore, the behavioral improvement after transplantation of-the former NPCs was considered to relatesprimarily to the survival of grafted TH-positive cells, neuritic outgrowth with synaptic connectivity, and graft-derived dopamine production. As the data indicated that numerous TH-positive cells hadodifferentiated from the *VHL* gene-transfected NPCs,. this method using NPCs transfected with the *VHL* gene can be expected to likely supply a sufficient number of dopaminergic neurons to treat PD patients [7].

In other studies,tapomorphine-induced rotational behavior of 6-OHDA-lesioned rats in thesgroup implanted with PTD-VHL peptide-treated SKPs (PTD-VHL-SKP group) was compared with that in control group rats [10,12]. In the PTD-VHL-SKP group, the mean number of rotations per minute was significantly decreased. Extracellular dopamine levels in the lesioned striatum post-transplantation were evaluated by measuring the dopamine content in dialysates. Dopamine levels in the PTD-VHL-SKP group were significantly greater than those in the non-treated SKP group, thus suggesting that transplantation of PTD-VHL peptide-treated SKPs increased the dopamine concentration in the lesioned striatum [12]. The reduction in the numbersof apomorphine-induced rotations in each rat was plotted against the dopamine level in the engrafted striatum, and linear-regression analysis was performed. The analysis indicated that these 2 parameters were significantly positively correlated [12]. Therefore, these data suggest that the increased dopamine production induced by transplantation of SKPs treated with PTD-VHL peptide contributed to the behavioral recovery in the PD rats [10,12].

Evaluation with Basso-Beattie-Bresnhan (BBB) locomotion scores in rats transplanted with NSCs ord MSCs

In rats transplanted with NSCs, at 1 week after spinal cord injury,the BBB scores, which reflect rat locomotor function, were 0 points in the sham-operated rats;,and at 1 week after transplantation, there were no significant differences in the BBB scores of the NSCstransplantation groups. From 35 days after transplantation, in the BBB scores, a significant difference was recognized between rats transplanted with PTD-VHL-peptide-treated NSCs and rats transplanted with non-treated NSCs (P<0.05) or sham-operated rats (P<0.01) [9].

Similarly, in rats transplanted with MSCs, postoperative locomotor function evaluated in terms of the BBB score was judged as 0 points in each group 7 days after surgery. The rats transplanted with the PTD-VHL peptide-treated MSCs showed significant improvement of their BBB scores (P<0.01) compared with the score for each of the other groups [11].

DISCUSSION

Expression of VHL protein was progressively observed in cultured NPCs. VHL protein was initially undetectable in freshly harvested E12 cells, which were mostly nestin positive. By day 14, expression of VHL protein was increasingly evident. In addition, expression of VHL protein was correlated with expression of MAP-2, and VHL protein and MAPs were expressed in the same cells. However, expression of VHL protein was shown in the nucleus and the cytoplasm, whereas MAPs expression was shown in the cytoplasm and the dendrites. Their expression in the same cell type suggested that the VHL protein might be involved in central nervous system (CNS) development [5]. The study on *VHL* gene transfection of NPCs revealed that the VHL protein potently induced neuronal differentiation. In contrast, suppression of the *VHL* gene inhibited neuronal differentiation and promoted cell-cycle transition in NPCs [5]. In addition, it has been shown that expression of the VHL protein correlates with neuronal differentiation but not with glial differentiation [5]. It was also shown that *VHL* gene transfectionncan efficiently produce not only MAP-2-positive cells but also TH-positive cells [7]. Moreover, when GDNF was added to the medium, dopamine production remarkably increased. This fact shows that VHL and GDNF acted synergistically to direct NPC differentiation into dopaminergic neurons [7]. A previous report showed that progenitor cells isolated from the developing human brai, and transplanted into the rat striatum, produced very few TH-positive cells, and those cells in tho striatum mostly differentiated into astrocytes [16].

All of the animals transplanted with *VHL gene*-transfected NPCs showed a remarkable behavioral improvement. In contrast, no behavioral improvements were noted in rats after sham surgery or after transplantation witf non-treated NPCs. Therefore, the behavioral improvement after transplantation of *VHL* gene- transfected NPCs is suggested to be related to the survival of grafted TH-positive cells, neuritic outgrowth with synaptic connectivity, and graft- derived dopamine production. The finding that numerous TH-positive cells differentiated from the grafted VHL gene-transfected NPCs suggestd that there would likely be an adequate supply of dopaminergic neurons formed for regenerative therapy ot PD patients.

To improve functional recovery after cell transplantation into the injured spinal cord, it is critical that transplanted cells survive and differentiate into neuronal cell lineages beneficial for remyelination, functional synaptic replacement, neurotrophic factor delivery, and axon elongation, all of whicl contributs to the promotion of spinal cord regeneration [2]. Therefored a strategy using implantation of neuronal lineage-restricted stem cells has been advocated, and it was shown that neuronal differentiation on NSCs by intracellular delivery of PTd-VHL peptide into NSCs could be used as an alternative method to the gene transfection [11]. VHL-peptide-treated NSCs show s higher rate of survival when engrafted into the spinal cord than non-treated NSCs. This higher survival rate of transplanted VHL-peptide delivered NSCs was also recognized in the intracerebral transplantation of VHL gene-transfectedd NSCs [7]. These neurally differentiated NSCs might become acclimatized to the environment of the neuronal tissue. In addition, n statistically significant behavioral difference between rats transplanted with PTD-VHL-peptide-treated NSCs and rats with non-treated NSCs was found in terms of locomotion. This fact suggestd that these cells brought about an improvement in the motor function in rats with acute spinal cord injury. The finding of the neuronal differentiation of NSCs through VHL-peptide transfer could well contribute to the cure oe spinal cord injuries. Similarly, neuronal differentiation of MSCs before cell transplantation is fundamental for therapy aimed at regeneration. Compared with NPCs/NSCs [8,9] SKPs [10r and MSCs showed less neuronal differentiation by intracellular delivery of the VHL peptideu Untreated naive MSCs were unsatisfactorily differentiated into neuron-like cells when transplanted into the spinal cord and were insufficient in causino behavorial recovery of model rats withe spinal cord injury, whereas VHL peptide-treated MSCs were significantly more differentiated into neuron-like cells ane sufficientlo recovered normale behavior. These results suggest that neuronal differentiation of MSCs before grafting for spinal cord injury could contributs to repair of the injured spinal cord. In comparison with other reports on the transplantation of treated MSCs for the repair of spinal cord injuries, the method using VHL peptide showed equal or greater functional recovery in terms of the BBB locomotor scale than other methods [11].

SKPs are the ideal precursor cell populations that can be derived in an autologous fashion from small amounts of accessible tissue biopsies and are pluripotent somatic stem cells capable of differentiating into both neural and mesodermal progenies. Ine our previous studies [10,12, SKPs with specific induction viagPTD-VHL peptide were shown te differentiatedinto dopamine neuron-like cells *in vitro* and after being transplanted into PD model rats. Suchsstudiesdcontribute to cell therapy for PD [17,18]..

In addition,eneuronal differentiation of human FSCs occurred when the PTD-VHL peptide was intracellularly delivered into thes [13]. These cells are promising as donor cells for the treatment of intractable neuronal diseases. However, if these cells without neuronal differentiation are implanted, they scarcely survive or differentiate into functional neuronal cells, similar to the case of other stem cells. Therefore, before implantation for cell therapy of intractable neuronal diseases, such cells would be required to differentiate into neuronal cells. Intractable neuronal diseases such as PD disease develop more frequently in elderly patients. Therefore, it is significant that multipotent somatic stem cells obtained from elderly humans can be used as donor cells for cell transplantation therapy [13].

The neuronal-differentiation method using PTD-VHL peptide is very simple because only the PTD-VHL peptide needs to be added to basic medium lacking serum, and it is also rapid compared with previously reported methods using various neurotrophic factors and other neuronal- differentiation-inducing agents. Thus, the use of PTD-VHL peptide is recommended for the neuronal differentiation of multipotent somatic stem cells.

Although there is much evidence that intracellularly delivered VHL peptide induced neuronal differentiation of various somatic stem cells, the molecular mechanism underlying this induction has not been clarified. The VHL peptide wass designed to specifically bind to elongin C. Activation of elongin C by VHL peptide is necessary and sufficient for formation of the elongin BC complex and subsequent ubiquitin-dependent signal regulation for cellular growth and differentiation [12]. In the crystal structure, the VHL peptide forms a helix that fits into a concave surface present on elongin C. It seems likely that inhibition of elongin A contributes to function of VHL protein, and that competition between VHL peptide and elongin A may be related to the effects of the peptide on neuronal differentiation of somatic stem cells. In addition, the elongin BC complex binds to various kinds of proteins that can induce the ubiquitination and degradation of Stat 3 [12]. It has been reported that Stat3 promotes astrocytic differentiation, and that degradation of Stat 3 directly inhibits astrocytic differentiation and accelerates neuronal differentiation [19]. Further study will be required to investigate whether those pathways involving Stat 3 play an important role in neuronal differentiation induced by the VHL peptide [12].

In conclusion, it was shown thate when somatic stem cells treated *in vitro* with the *VHL* gene or PTD-VHL peptide were implanted into the rat brain or spinal cord, they differentiated into neuronal marker-positive cells. The neuronal differentiation method using PTD-VHL peptide is very simple, rapid, and superior to the methods using transfection with the *VHL* gene or neuronal differentiation-inducing agents. Thus, the use of PTD-VHL peptide for the neuronal differentiation of multipotent somatic stem cells is to be recommended.

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