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TOPIC HIGHLIGHT

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Diagnosis and management of pancreatic neuroendocrine tumor in von Hippel-Lindau disease

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Abstract

The pancreatic manifestations seen in patients with von Hippel-Lindau (VHL) disease are subdivided into 2 categories: pancreatic neuroendocrine tumors (NET), and cystic lesions, including simple cyst and serous cystadenoma. The VHL-associated cystic lesions are generally asymptomatic and do not require any treatment, unless they are indistinguishable from other cystic tumor types with malignant potential. Because pancreatic NET in VHL disease are non-functioning and have malignant potential, it is of clinical importance to find and diagnose these as early as possible. It will be recommended that comprehensive surveillance using dynamic computed tomography for abdominal manifestations, including pancreatic NET, should start from the age of 15 years in VHL patients. Unlike sporadic non-functioning NET without VHL disease, in which surgical resection is generally recommended, VHL patients at lower metastatic risk of pancreatic NET should be spared the risks of operative resection.

Key words: Von Hippel-Lindau disease; Pancreas; Neuroendocrine tumor, Diagnosis; Clinical protocols

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INTRODUCTION

Von Hippel-Lindau (VHL) disease is an autosomal dominant disorder that develops a variety of tumors and cysts in the central nervous system (CNS) and visceral organs[1]. The prevalence of patients with VHL disease was reported to be 1 in 100000 of the population and 1 family in 1 million of the population[2]. Tumor types seen in VHL disease include hemangioblastomas in the CNS and retina, renal cell carcinoma, pheochromocytomas and pancreatic neuroendocrine tumors (NET)[1]. During their growth, these tumors impair the function of the primary organs and sometimes metastasize to distant organs, and thus are thought to have malignant potential. A number of studies in the United States and Europe have reported the clinical characteristics of these tumors, including pancreatic NET[3-9].

PANCREATIC MANIFESTATIONS IN VHL DISEASE

The pancreatic manifestations seen in patients with VHL disease are subdivided into 2 categories: NET as solid tumors, and cystic lesions, including a simple cyst and serous cystadenoma[1,5,10]. Fortunately, cystic lesions complicated with VHL disease are generally asymptomatic and do not require any treatment (Figure 1)[1]. It is necessary to differentially diagnose them from other cystic tumor types, such as intraductal papillary mucin-producing tumors or mucinous cystic tumors, because these mucinous cystic tumors have malignant potential. When cystic lesions seen in patients with VHL disease are indistinguishable from these tumor types or are causative of compression symptom onto adjacent organs, operative resection of the cystic lesion in the pancreas would be considered.

Unlike cystic lesions seen in the pancreas of patients with VHL disease, NET can be locally invasive and can metastasize, resulting in much higher clinical significance[1,6]. NET occur in 8%-17% of patients with VHL disease[1]. The malignant potential of sporadic pancreatic NET, which is not associated with VHL disease, varies depending on the functional properties of the tumors. None of the patients with pancreatic NET associated with VHL disease has been reported to present with hormonal syndrome[3,8]. Sporadic non-functioning NET behave in a malignant fashion with a metastatic spread in 60% to 90%, in marked contrast to the findings in cases with pancreatic NET associated with VHL disease, as previously described (metastatic disease in 11%-20%)[11]. The reason is thought to be as follows. In the case of sporadic non-functioning pancreatic NET, there are no hormonal symptoms, hence the tumors are first identified when they grow larger than 5 cm. In contrast, in the case of pancreatic NET in patients with VHL disease, the tumors can be diagnosed at a relatively early stage by screening examination for abdominal manifestations of the disease[11].

In general, pancreatic NET with or without VHL disease show a slow growth phenotype and thus the patients have a good prognosis. Blansfield *et al*[12] reported that the death rate as a result of metastatic pancreatic NET was 0.3% in patients (n = 633) with VHL disease. Pancreatic NET tend to have a high frequency in patients with pheochromocytoma (VHL type 2) as previously described[3,11]. However, Hammel *et al*[5] reported that patients with pancreatic lesions had significantly fewer pheochromocytomas than those without pancreatic lesions (14/122 *vs* 16/36, $P < 0.0001$). Taken together, there is no consensus to date regarding coexistence of pancreatic NET and pheochromocytoma.

DIAGNOSIS OF PANCREATIC NET IN VHL DISEASE

Ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI) can be used to detect primary NET and their metastases. Octreotide scintigraphy has a sensitivity that exceeds the combination of the others. However, smaller tumors can be difficult to visualize with octreotide scintigraphy. Positron emission tomography with 5-hydroxytryptophan or L-dopa can be an option for detection of small tumors[13], although only a limited number of institutes have employed these methodologies. In almost all hospitals over the world, dynamic CT is the most sensitive method for detection at present, since pancreatic NET are strongly enhanced on dynamic CT (Figure 2)[14]. MRI is also an effective method for metastatic liver lesions[15].

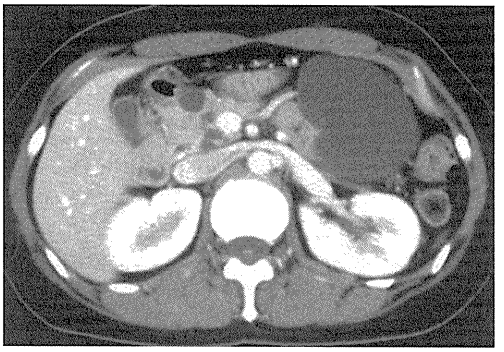

Figure 1 Abdominal computed tomography shows several cystic lesions in the pancreas (32-year-old female).

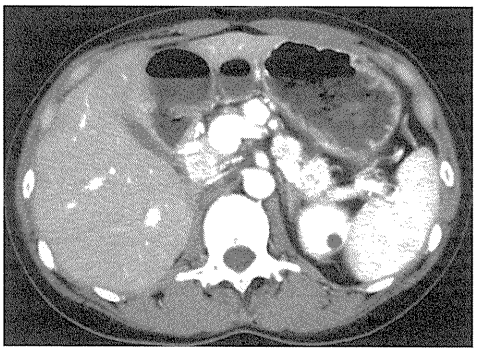

Figure 2 Contrast-enhanced abdominal computed tomography reveals several pancreatic mass lesions that are strongly enhanced (33-year-old female)[14].

MANAGEMENT OF PANCREATIC NET IN VHL DISEASE

In past reports, the youngest age at diagnosis of pancreatic NET in patients with VHL disease is 12 years old[16], and the second youngest age is 16 years old[12]. The surveillance of renal cell carcinoma (RCC) in VHL disease has been begun from the age of 15, therefore it will be recommended that comprehensive surveillance of abdominal organs including pancreas starts from the age of 15 by abdominal dynamic CT in view of the risk from

Table 1 Treatment recommendations for pancreatic neuroendocrine tumors with von Hippel-Lindau disease[12]

	Treatment recommendation
Prognostic criteria	
Tumor size ≥ 3 cm	
Mutation in exon 3	
Tumor doubling time ≤ 500 d	
None of the criteria	Followed by CT/MRI every 2-3 yr
1 criterion	Followed by CT/MRI every 6-12 mo
2 or 3 criteria	Consider surgical intervention

CT: Computed tomography; MRI: Magnetic resonance imaging.

radiation exposure and renal dysfunction caused by contrast media. In addition, patients with VHL disease require particular attention to distinguish pancreatic NET from metastatic RCC, because pancreatic metastasis from RCC is visualized as a hypervascular tumor as well as pancreatic NET. If pancreatic NET are not found by dynamic CT in the first abdominal surveillance (at the age of 15 years), the patient can be followed with comprehensive surveillance including that for RCC and pheochromocytoma every 2-3 years[12].

Sporadic non-functioning NET without VHL disease behave in a malignant fashion, therefore surgery is recommended to avoid later development of malignancy in all cases with tumor size greater than 2 cm[17,18]. In contrast, in the case of pancreatic NET with VHL disease, the indication for surgery should be carefully decided, because the patients commonly have multiple or recurrent tumors. The problem of surveillance is how to manage pancreatic NET without metastasis.

Blansfield *et al*[12] proposed 3 criteria to predict metastatic disease of pancreatic NET in patients with VHL disease: (1) tumor size greater than or equal to 3 cm; (2) presence of a mutation in exon 3; and (3) tumor doubling time less than 500 d (Table 1). If the patient has none of these criteria, they suggested that the likelihood of the patient's lesion resulting in metastatic disease is very low and that the patient can be followed with a medical history and physical examination and radiologic surveillance on 2-3 years cycles. If the patient has 1 criterion, the patient should be followed more closely every 6 mo to 1 year to detect the emergence of a second criterion. If the patient has 2 or 3 criteria, the patient should be considered for surgical management because of the greater likelihood of future malignancy from pancreatic NET[12]. The treatment strategy in patients with the metastatic disease is still controversial, depending on histological tumor types.

CONCLUSION

It is of clinical importance to find and diagnose pancreatic NET in patients with VHL as early as possible. It is recommended that comprehensive surveillance for abdominal manifestations in VHL patients including pancreatic NET should start from the age of 15. In general, pancreatic NET with or without VHL disease show a slow growth phenotype and patients have a good prognosis. VHL patients at lower metastatic risk from pancreatic NET should be spared the risks of surgical resection.

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Engrafted VHL peptide-delivered bone marrow stromal cells promote spinal cord repair in rats

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Stem cell-based therapy using bone marrow stromal cells (MSCs) has been expected to be a promising therapy for neuronal regeneration. To repair the injured spinal cord, neuronal differentiation of MSCs before transplantation has a more satisfactory effect. Recently, neuronal differentiation of neural progenitor/stem cells by an intracellular delivery of a pVHL-derived synthetic peptide (VHL peptide) has been shown. Here, we show that VHL peptide-delivered MSCs differentiated into neuron-like cells, and that engrafted VHL peptide-delivered MSCs more recovered the behaviors of the rats than that of nondelivered MSCs. Our result suggests that the use of VHL peptide-delivered MSCs would be a promising therapeutic strategy for repairing the injured spinal cord. *NeuroReport* 21:287–292 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: bone marrow stromal cells, neuronal differentiation, spinal cord injury, transplantation, VHL peptide

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Introduction

Cell-based therapy using multipotent stem cells has been recognized as an attractive and promising method for the treatment of spinal cord injury. Bone marrow stromal cells (MSCs) are easily isolated and can be used for autologous transplantation, avoiding immune problems. MSCs are able to differentiate into cells of neural lineages; however, the main problem is that only a small number of cells become functional neurons [1]. There have been several reports of neuronal differentiation of MSCs by using neurotrophic factors [2,3] or by gene transfer [4–6], which is associated with risks such as viral toxicity and cancer formation. We showed earlier that the VHL protein had the potential to transform neural progenitor cells into neurons *in vitro* [7]. Recently, intracellular delivery of a synthetic oligopeptide derived from the VHL protein (VHL peptide) showed neuronal differentiation in neural progenitor/stem cells and skin-derived precursors [8–10], and transplantation of VHL peptide-delivered neural stem cells promoted recovery in the injured rat spinal cord [9]. In this study, we showed that intracellular delivery of VHL peptide induced neuronal differentiation in MSCs. Moreover, we transplanted VHL peptide-delivered MSCs into the injured rat spinal cord and showed repair of the spinal cord injury with the evaluation of functional recovery of the motor function.

Materials and methods

Isolation and culture of bone marrow stromal cells

Primary rat MSCs were isolated according to the method described earlier [4]. Briefly, they were obtained from the femurs and the tibias of 6-week-old male Wistar rats by flushing out the bone marrow with α-minimum essential medium (Gibco-BRL, Grand Island, New York, USA), and the cells were seeded into culture flasks in α-minimum essential medium containing 10% fetal bovine serum, and then cultured in a CO_2 incubator. Subsequently, the culture flasks were left undisturbed for 4–5 days to promote cell attachment. Later, the nonadherent cells were removed. At near-confluence, the cells were subcultured after trypsin digestion and passaged four times. These cells were then used in the experiments.

Peptide synthesis

As described earlier [8–10], we chemically synthesized a peptide corresponding to the 157–171 amino acid sequence in the amino acid region of the pVHL. The synthesized peptide was linked with the protein transduction domain of the HIV-TAT protein [TATVHL(157–171) peptide], thereby facilitating peptide entry into the cells. Control peptides containing only the protein transduction domain of the TAT protein [TAT peptide], or the 104–123 amino acid sequence of the β-domain in the VHL protein [TATVHL(104–123) peptide] were synthesized [11]. Peptide complexes were also labeled with FITC to observe the cellular internalization of the peptide. These synthesized peptides are as follows:

TATVHL(157–171), NH_2-YGRKKRRQRRRDTLKERCLQVVRSLVK-COOH; TATVHL(104–123), NH_2-YGRKRRQRRRDGTGRRIHSYRGHLWLFRDAG-COOH; TAT, NH_2-YGRKKRRQRRRD-COOH.

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Neuronal differentiation of bone marrow stromal cells

MSCs were subsequently incubated in DMEM/F12(1 : 1) (Gibco-BRL) containing a N2 supplement (Gibco-BRL) and 1 μM peptide was delivered into the MSCs for neuronal differentiation. The protocol of the *in vitro* study was the same as the transplantation study.

Spinal cord injury model and transplantation of MSCs

A total of 36 young and adult male Wistar rats weighing between 200 and 250 g were used in this study. The rats were equally divided into the following four groups: a control group, in which only a sham operation was performed; a group transplanted with non-treated MSCs; one transplanted with TATVHL(104–123)-treated MSCs; and one transplanted with TATVHL(157–171) peptide-treated MSCs. The rats were anesthetized with isoflurane and then the lamina of the 10th thoracic vertebra was removed. Spinal cord injury was inflicted by using an injury device (Pneumatic Injury Device PiD-1000, Physio-Tech, Tokyo, Japan). On day 7, after the spinal cord injury, MSCs were transplanted into the damaged spinal cord. Before transplantation, MSCs were pre-labeled with red fluorescence PKH26PCL (Sigma, St. Louis, Missouri, USA). The experimental groups received 1×10^5 MSCs in a 10-μl volume. Control rats received an intraspinal injection of 10 μl of the culture medium alone. The locomotor function was evaluated by using the Basso–Beattie–Bresnahan (BBB) Locomotor Rating Scale [12] every week until 6 weeks after the transplantation. All surgical interventions and animal care were carried out in accordance with the Laboratory Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals, and were approved by the Animal Ethics Committee of the Yokohama City University School of Medicine.

Immunocytochemistry and immunohistochemistry

Cells or segments were incubated for 1 h at room temperature with primary antibodies. Antibodies against β-tubulin (Tuj-1; R&D Systems; Minneapolis, Minnesota, USA), neurofilament-200 (NFH; Sigma), and microtubule-associated protein-2 (MAP-2; Sigma) were used. The thin sections of the spinal cords were rinsed in PBS and then incubated for 1 h with secondary antibody, either rhodamine-conjugated anti-mouse IgG or anti-rabbit IgG (Sigma). Nuclear counterstaining was done with TOPRO-3 (Molecular Probes; Eugene, Oregon, USA) or DAPI (Molecular Probes), and observations were made with a fluorescence microscope system (FV300, Olympus, Tokyo, Japan). To assess the frequency of different cell types, we counted the number of cells that were immunopositive with a given antibody in 10–15 random nonoverlapping visual fields (50–200 cells per field) in each experiment. At least three experiments were performed for each condition. The degree of positivity was expressed as the ratio of immunopositive cells to the total number of nuclei stained with TOPRO-3 or DAP1. Positive controls were used PKH26PCL-labeled neuronal cells and negative controls used non-labeled neuronal cells without a primary antibody.

Western blotting

Cultured cells were washed three times in cold PBS and then scraped into ice-cold PBS. After incubation on ice for 10 min, the cells were lysed with a lysis buffer [8], and then were centrifuged and supernatants were collected. Each sample was separated by SDS-PAGE under reducing conditions and transferred electrophoretically to nitrocellulose filters. Nonspecific binding of antibody was blocked by overnight incubation with 5% skim milk. Western blots were probed with rabbit anti-NFH and mouse anti-MAP-2 followed by horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected by using a chemical luminescence detection system (ECL Plus Western Blotting Reagent Pack, Amersham, Hemel Hempstead, UK). Images were analyzed with LAS-1000 (Fujifilm, Tokyo, Japan), and the density of the bands was determined by using Image Gauge software (Fujifilm, Tokyo, Japan).

Reverse transcriptase polymerase chain reaction

Total RNA was extracted from cultured MSCs with TRIzol reagent (Life Technologies Inc., Carlsbad, New York, USA). RNA was converted to cDNA using a TaKaRa one-step RNA PCR Kit (Takara Bio, Otsu, Japan). PCR was conducted by using the Ex Taq Hot-start version (Takara Bio) with random hexamer primers. The synthesized cDNA was amplified under the following conditions: initial denaturation at 94°C, 5 min; denaturation at 94°C, 1 min; annealing at 57–59°C, 1.5 min; and extension at 72°C, 1.5 min for 25–35 cycles. The sequences of primers were as follows: 5′-AAGTACCATTTAGGCATGAGCT C-3′ (forward) and 5′-AATCAAGGCAAGACATAGCGA-3′ (reverse) for MAP2, 5′-AGAAGCACTTGGTTTTATT GCAC-3′ (forward) and 5′-GGCTTTGGTCCATCTCAA CAAAC-3′ (reverse) for neurofilament 200, and 5′-TTGT AACCAACTGGGACGATATGC-3′ (forward) and 5′-ATA GCTGTGCAACTTGTCTGGTGC-3′ (reverse) for β-actin.

Statistical analysis

Results were expressed as mean ± standard deviation. For comparisons between values for groups, the Scheff test after the ANOVA test was used, with probabilities of less than 0.05 being considered significant (Statcel version 5.0/7.0, California, USA).

Results

Immunocytochemical results

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 MAP2. TATVHL(157–171) peptide-delivered MSCs showed significantly higher rates in expressions of both Tuj-1 and NFH than TATVHL(104–123) peptide-delivered MSCs ($P < 0.05$), TAT peptide-delivered

MSCs ($P < 0.01$), or nontreated control cells ($P < 0.01$). TATVHL(104–123) peptide-delivered MSCs also showed significantly higher rates in expressions of both Tuj-1 and MAP2 than TAT peptide-delivered MSCs or control cells ($P < 0.01$) (Fig. 1).

Reverse transcriptase polymerase chain reaction and western blotting results

In the reverse transcriptase polymerase chain reaction experiments done to analyze the expression of factors related to neural development, β-actin served as an internal control. NFH and MAP2 mRNAs were detected by reverse transcriptase polymerase chain reaction in RNA prepared from MSCs treated with TATVHL(157–171) or TATVHL(104–123) peptide, while they were significantly less detected in MSCs treated with TAT peptide ($P < 0.01$). The quantitative evaluation indicated that the TATVHL(157–171) peptide had more expression in mRNA of NFH or MAP2 than the TATVHL(104–123) peptide ($P < 0.05$).

In line with mRNA expression, western blots of TATVHL (157-171) or TATVHL(104–123) peptide-treated MSCs showed a distinct presence of NFH and MAP2, whereas that of TAT peptide-treated MSCs scarcely did. Quantitative evaluation indicated that the TATVHL(157–171) peptide had significantly more neuronal differentiation induction activity than the TATVHL(104–123) peptide ($P < 0.05$) (Fig. 2).

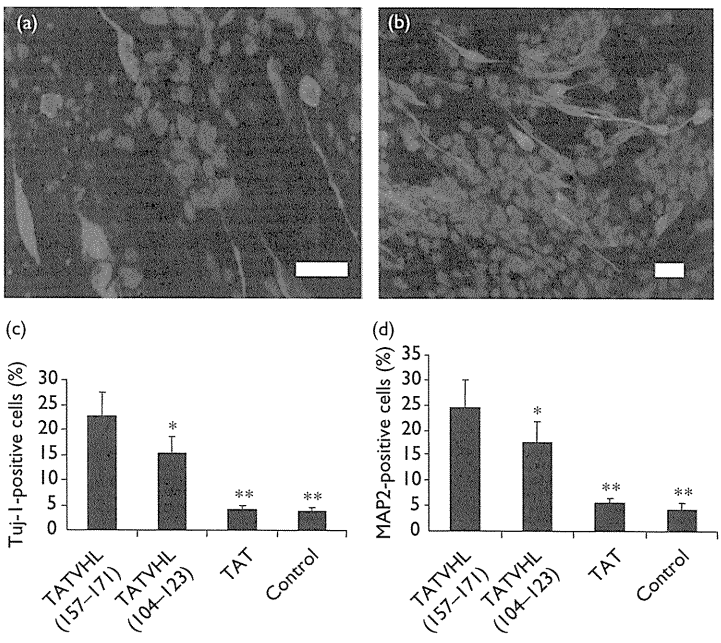
Basso-Beattie-Bresnahan locomotion scores

Postoperative locomotor function evaluated in terms of the BBB score was judged as 0 points in each group 7 days after surgery. Seven weeks after spinal cord injury (6 weeks after transplantation), the BBB score differed among the groups: 6.0 ± 0.5 ($n = 9$), sham operated; 8.6 ± 0.5 ($n = 9$), rats transplanted with nontreated MSCs; 9.5 ± 0.5 ($n = 9$), rats transplanted with TATVHL(104–123) peptide-delivered MSCs, and 11.2 ± 0.7 ($n = 9$), rats transplanted with TATVHL(157–171) peptide-delivered MSCs. The rats transplanted with the TATVHL(157–171) peptide-delivered MSCs showed significant improvement of their BBB scores ($P < 0.01$) compared with the score for the other groups (Fig. 3).

Immunohistochemical results

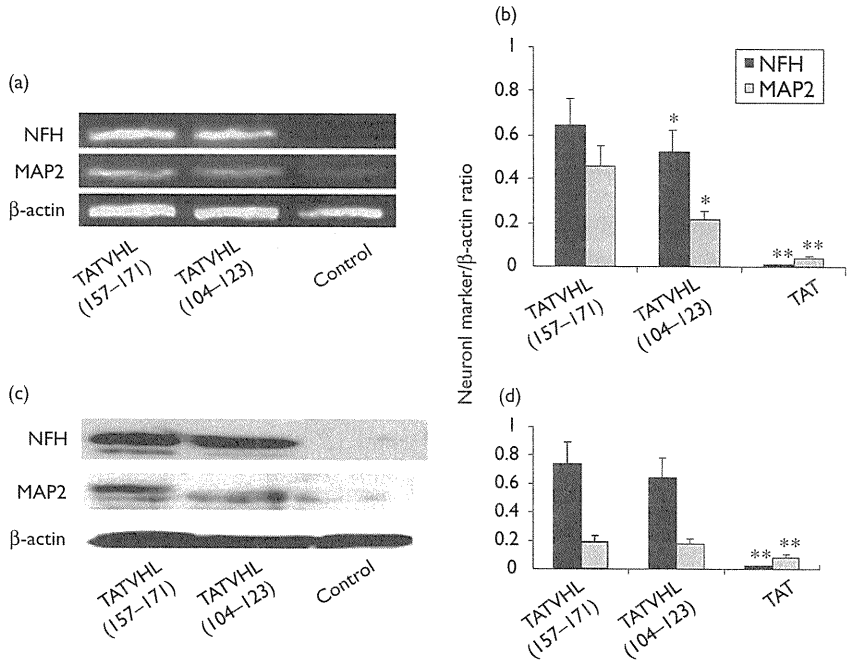
Four weeks after transplantation of MSCs, confocal analysis of MSC differentiation in TATVHL(157-171) peptide-delivered MSCs or TAT peptide-delivered MSCs showed that the number of cells that are survived (red fluorescence PKH-prelabeled cells) of TATVHL (157–171) peptide-delivered MSCs ($10.4 \pm 1.7\%$) is significantly

Fig. 1



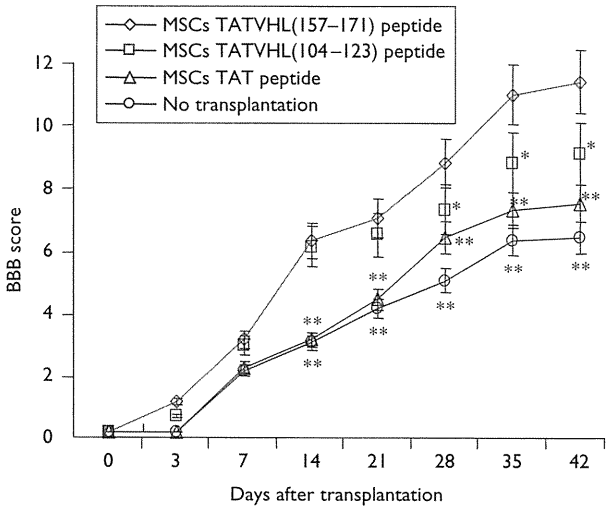
Immunocytochemical study for bone marrow stromal cells (MSCs) after the treatment with peptides. Immunocytochemical analysis of Tuj-1 (a) or MAP2 (b) for MSCs 7 days after treatment with TATVHL(157–171) peptide. The cells were labeled with the nuclear stain YoYo-1 (blue), and immunoreactive cells showed green. Rate of cells showing immunoreactive for anti-Tuj-1 antibody (c) or anti-MAP2 antibody (d) at 7 days after treatment with TATVHL(157–171) peptide, TATVHL(104–123) peptide, or TAT peptide. *$P<0.05$; **$P<0.01$ in significant difference between TATVHL(157–171) and TATVHL(104–123) or TAT. Scale bar=10 μm.

Fig. 2



Western blotting (a and b) and reverse transcriptase polymerase chain reaction analyses (c and d) for NFH and MAP2 in TATVHL(157–171) peptide-treated, TATVHL(104–123) peptide-treated, or TAT peptide-treated bone marrow stromal cells (MSCs) 7 days after the treatment. The results show TATVHL(157–171)-peptide-treated MSCs show significantly greater expression of NFH and MAP2 than TATVHL(104–123) or TAT peptide-treated MSCs at both mRNA and protein levels. *$P<0.05$; **$P<0.01$ in significant difference between TATVHL(157–171) and TATVHL(104–123) or TAT.

Fig. 3



Basso–Beattie–Bresnahan (BBB) scores for assessing recovery of behavioral performance after spinal cord injury in the various groups. The BBB score was significantly improved for the rats transplanted with TATVHL(157–171) peptide-treated bone marrow stromal cells (MSCs) compared with the score for the nontransplantation control. *$P<0.05$; **$P<0.01$ in significant difference between TATVHL(157–171) and TATVHL(104–123) or TAT.

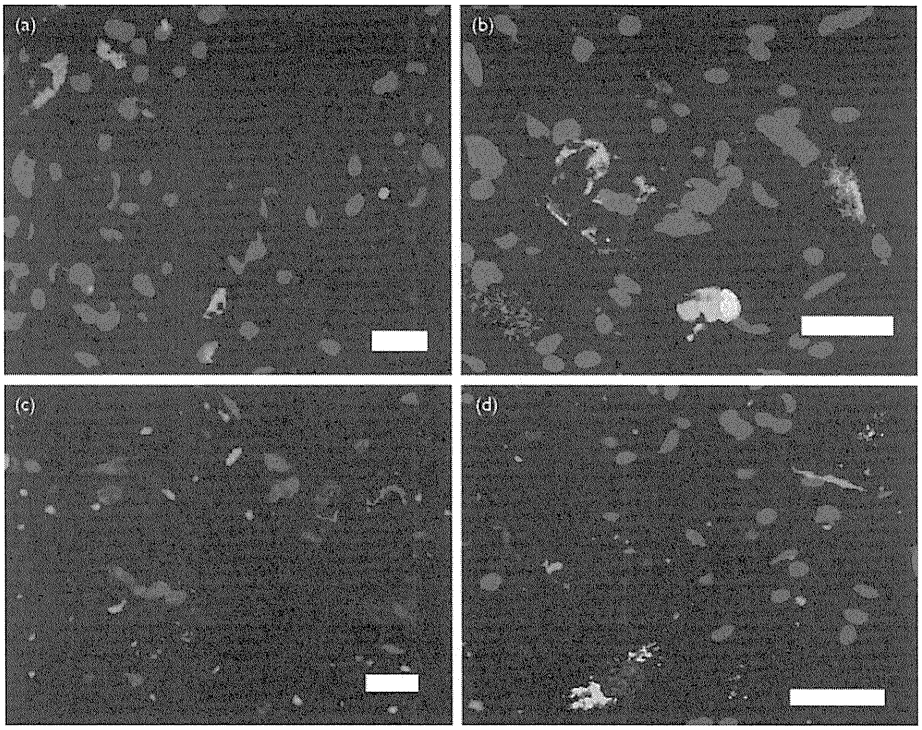
greater than that of TAT peptide-delivered cells ($2.3 \pm 0.6\%$) ($P < 0.01$). In addition, PKH-prelabeled MSCs had co-localized with NFH in $28.8 \pm 3.5\%$ of TATVHL(157–171) peptide-delivered cells whereas those did with NFH in $6.3 \pm 1.4\%$ of TAT peptide-delivered cells, indicating that TATVHL(157-171) peptide MSCs had differentiated more into NFH-positive cells than TAT peptide-delivered MSCs (Fig. 4).

Discussion

Neuronal differentiation of MSCs before cell transplantation is fundamental for therapy aimed at regeneration. In this study, we proposed a novel method using intracellular delivery of VHL peptide for neuronal differentiation in MSCs. Compared with neural progenitor/stem cells [8,9] or skin-derived precursors [10], MSCs showed less neuronal differentiation by intracellular delivery of VHL peptide. Although the mechanism of neuronal differentiation by intracellular delivery of VHL peptide is unclear, the response to VHL peptide might be different in cell types.

Our transplantation experiment has shown that untreated naive MSCs were unsatisfactorily differentiated into neuron-like cells and were insufficient to recover the behaviour of the spinal cord injury model rats, whereas VHL peptide-delivered MSCs were significantly more

Fig. 4



Confocal immunohistochemical images of nontreated or treated bone marrow stromal cells engrafted in injured rat spinal cord sites. The transplanted cells were prelabeled with red fluorescence PKH. The cells were stained with cell-specific markers and their nuclei were stained with DAPI. (a) Image of NeuN (green), PKH (red), and DAPI (blue) in the nontreated cells engrafted tissue. (b) Image of NeuN in the treated cells engrafted tissue. (c) Image for NFH (green), PKH (red), and DAPI (blue) in the nontreated cells engrafted tissue. (d) Image for NFH (green), PKH (red), and DAPI (blue) in the treated cells engrafted tissue. Scale bar = 10 μm.

differentiated into neuron-like cells and were sufficient to recover the behaviour. These results suggest that neuronal differentiation of MSCs before grafting for spinal cord injury contributes to repair of the injured spinal cord. In comparison with other reports on the transplantation of treated MSCs for spinal cord injury repair, the method we used with VHL peptide showed equal or greater functional recovery in the BBB locomotor scale [6,13].

In conclusion, neuronal lineage marker-positive cells are efficiently generated from MSCs with intracellular delivery of the TATVHL(157–171) peptide, and also that the engrafted TATVHL(157–171) peptide-delivered MSCs restore the physiological function in the spinal cord injury model rats. This study provides a promise of clinical application of autologous donor cells derived from readily accessible bone marrow for the cell-transplantation therapy for patients with spinal cord injury.

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