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Neuropsychobiology; 2005; 51, 1; ProQuest Medical Library
pg. 34

Original Paper

Neuropsychobiology

Neuropsychobiology 2005;51:34–38
DOI: 10.1159/000082853

Published online: December 20, 2004

Anxiolytic Effect of Hepatocyte Growth Factor Infused into Rat Brain

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Key Words
Anxiety · Hepatocyte growth factor · Rat

Abstract
Background: Hepatocyte growth factor (HGF) has the capacity to selectively direct thalamocortical projections into an intermediate target, the pallidum, and eventually to their final cortical destination. HGF may have a role in the mediation of anxiety. Very little is known about other central behavioral effects of HGF. ***Objective:*** Our aim was to determine what effect HGF has on anxiety in rats. ***Methods:*** HGF was infused at a constant rate into cerebral lateral ventricles and its effect on anxiety in rats was monitored. ***Results:*** In the elevated plus maze test and the black and white box test, HGF administration caused all indicators of anxiety to increase. No significant effect on general locomotor activity was seen. ***Conclusion:*** HGF infusion into the brain produces an anxiolytic effect.

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Introduction

Hepatocyte growth factor (HGF) is a potent angiogenic growth factor [1–3]. Recently, it has been reported that HGF is induced in neurons during ischemia [4] and that HGF is neuroprotective against postischemic delayed neuronal death in the hippocampus [5, 6].

In the brain, HGF is expressed by specific classes of neurons in addition to nonneuronal cells in the ependyma and choroid plexus [7]. In contrast to HGF, c-Met transcripts have been predominantly localized in neurons of the cerebral cortex, hippocampus and septum [8–10]. HGF elevated the proto-oncogene *c-fos* mRNA in cultured septal neurons, showing a functional interaction between c-Met and its ligand [10]. This result, together with the presence of c-Met in the developing brain, raised the possibility that HGF may have a neurotrophic activity on central neurons. In keeping with this hypothesis, Hamanoue et al. [11] showed that HGF promoted the survival of cultured mesencephalic tyrosine hydroxylase-positive neurons. HGF acts on calbindin-D-containing hippocampal neurons and increases their neurite outgrowth, suggesting that HGF plays an important role in the maturation and function of hippocampal neurons [12]. Transfection of HGF gene into the subarachnoid space prevent-

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0302–282X/05/0511–0034$22.00/0

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ed delayed neuronal death, accompanied by a significant increase in HGF in the cerebrospinal fluid (CSF). Prevention of delayed neuronal death by HGF is due to the inhibition of apoptosis through the blockade of bax translocation from the cytoplasm to the nucleus. HGF gene transfer into the subarachnoid space may provide a new therapeutic strategy for cerebrovascular disease [13].

HGF has the capacity to selectively direct thalamocortical projections into an intermediate target, the pallidum, and eventually to their final cortical destination [14]. Mice with a targeted mutation of the gene encoding urokinase plasminogen activator receptor (uPAR), a key component in HGF/scatter factor (SF) activation and function, have decreased levels of HGF/SF and a 50% reduction in neocortical GABAergic interneurons at embryonic and perinatal ages. Mice of the uPAR –/– strain survive until adulthood, and behavior testing demonstrates that they have an increased anxiety state [14]. HGF may have a role in the mediation of anxiety.

This is the first report to determine what effect HGF infused into cerebral lateral ventricles has on anxiety in rats.

Materials and Methods

Animals

Five-week-old male Wistar rats (Seack Yoshitomi Co., Fukuoka, Japan) were used for the present study. The number of rats was each 10 rats for experimental and control groups. The rats were housed in pairs for 3 weeks prior to the start of behavioral experiments in a sound-proof room at 24 ± 0.5°C, 50 ± 5% relative humidity, with controlled 12-hour light-dark cycles (light from 18:00 to 6:00), and were allowed free access to food and water. The room was cleaned at random in a dim, red light. All testing was performed in July during the dark phase using a dim, red light. Animal care was in accordance with the guidelines for animal experimentation of Oita Medical University.

Surgical Procedures

Each rat was anesthetized with chloral hydrate (400 mg/kg, i.p.), a brain infusion cannula (brain infusion kit, model 1007D, Alzet Corp., Palo Alto, Calif., USA) was stereotactically implanted into the lateral cerebral ventricle (0.92 mm caudal and 1.6 mm lateral to the bregma and 3.5 mm deep), and a mini-osmotic pump (micro-osmotic pump, model 1003D; Alzet Corp.) was placed into subcutaneous tissue of the back. After the operation, rats were injected with ceftriaxone sodium (20 mg/kg, i.p.). Either HGF (30 μg) in the experimental group or a vehicle in the control group (Ringer's solution, pH = 7.4) was infused at a constant rate into the lateral ventricle of the rat via the micro-osmotic pump over a 3-day period. Tsuzuki et al. [15] reported that continuous intraventricular administration of the human recombinant HGF by using an osmotic mini-pump reduced the infarct volumes in the brain lesion and prevented apoptotic neuronal cell death.

Materials

A vehicle (Ringer's isotonic solution, pH 7.4) was used as a control. HGF was synthesized in the Division of Biochemistry, Department of Oncology, Biomedical Research Center, Osaka University Medical School.

Behavioral Testing

The first day of testing was concerned with measuring anxiety. All rats were subjected to the 'elevated plus maze', followed on the same day by the 'black and white box' test. Ethological measures in elevated plus maze comprised frequency scores for supported head dipping (exploratory movement of head/shoulders over the side of the maze), and stretched attend posture (exploratory posture in which the body is stretched forward then retracted to the original position without any forward locomotion). At the end of the day, rats received inescapable electric foot shocks to condition fear. On the second day, rats performed the conditioned fear test. Conditioned response models of fear and anxiety are based on classical procedures of fear conditioning [16]. On day 1 of fear conditioning, each rat was individually subjected to 5 min of inescapable electric foot shock (10 shocks of 1 s duration and 2 mA intensity, each shock separated by an interval of 40 s) in a chamber with a grid floor (31 × 30 × 25 cm). Twenty-four hours after the foot shock, the rats were again placed in the shock chamber and observed for 5 min without shocks. During the 5-min observation period, freezing behavior was recorded using a video camera. Every 10 s, the behavior was classified as either freezing or active. Freezing was defined as the absence of any observable movement of the body and/or vibrissae, aside from the movement necessitated by respiration. We also investigated general locomotor activity.

Elevated Plus Maze

The elevated plus maze consisted of two opposite open arms (50 × 10 cm) without side walls and two opposite enclosed arms (50 × 8 × 40 cm), and was elevated 50 cm above the floor. The rats were placed in the middle of the maze facing one of the open arms, and immediately left alone in the test room. They were observed and their responses were recorded for 300 s via a video camera. Five parameters were measured during 5 min: (1) time spent in the open arms, (2) total number of entries into the open arms, (3) number of stretched attend postures, and (4) number of head dips over the edge of the platform.

Black and White Box

The wall of the test box was 27 cm high, the size of each compartment was 23 × 27 cm, and the two compartments were connected by a 10-cm high semicircular hole. Both white and red light sources were 40 W, and the light sources were located 17 cm above the floor of the two compartments. The rats were placed in the center of the white compartment and the number of entries and time spent in the black and white compartments during 5 min were recorded. An entry into another compartment was scored whenever a rat placed all four paws in that compartment.

General Locomotor Activity

We investigated general locomotor activity of the rats by means of infrared photobeam breaks, since locomotion influences exploratory activity. The apparatus was 36 cm in height and the floor size was 30 × 30 cm. We measured the locomotor activity by photobeam breaks for 2 h.

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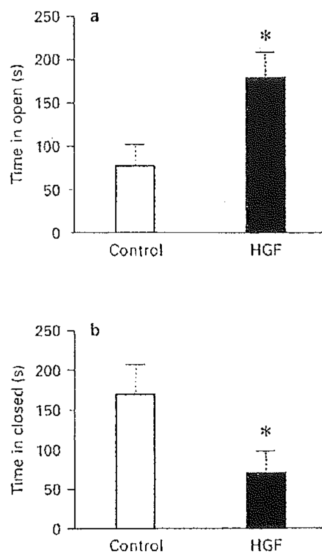

Fig. 1. a Time spent in the open arm of the elevated plus maze was significantly increased in the HGF-infused group compared to the control group. **b** Time spent in the closed arm of the elevated plus maze was significantly decreased in the HGF-infused group compared to the control group. * p < 0.05 vs. vehicle- and HGF-infused group.

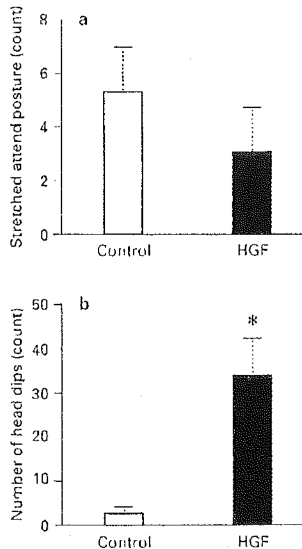

Fig. 2. a No effect of HGF or the control vehicle on the number of stretched attend postures in the elevated plus maze was seen. **b** The number of head dips in the elevated plus maze was significantly increased in the HGF-infused group compared to the control group. * p < 0.05 vs. vehicle- and HGF-infused group.

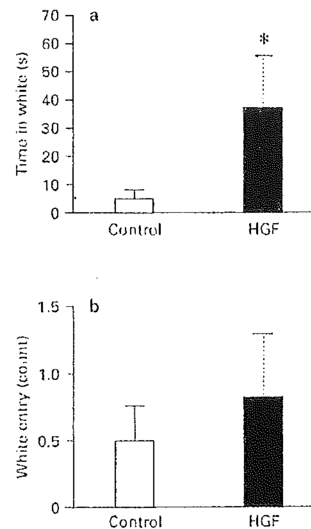

Fig. 3. In the black and white box test, the time spent in the white chamber was significantly increased in the HGF-infused group, compared to the controls (**a**). **b** No significant effect of HGF on the number of white chamber entries was seen. * p < 0.05 vs. vehicle- and HGF-infused group.

Statistical Analysis

The data were presented as means ± SE of the individual values from each group. Behavioral data (except for general locomotor activity) were analyzed using the Student t test for independent samples. The data of general locomotor activity were subjected to a two-way ANOVA. Statistical significance was accepted for $p < 0.05$.

Results

Time spent in the open arm of the elevated plus maze was significantly increased in the HGF-infused group, compared to the vehicle-treated group [$t(18) = 2.43$, $p < 0.031$; fig. 1a]. Time spent in the closed arm of the elevated plus maze was significantly decreased in the HGF-infused group compared to the control group [$t(18) = 2.23$, $p < 0.045$; fig. 1b]. No effect of HGF or the control vehicle on the number of stretched attend postures in the elevated plus maze was seen [$t(18) = 1.01$; fig. 2a]. The number of head dips in the elevated plus maze was significantly increased in the HGF-infused group compared to the control group. The number of head dips was significantly increased in the HGF-infused group compared to the vehicle-treated group [$t(18) = 2.61$, $p < 0.023$; fig. 2b]. In the black and white box test, the time spent in the white chamber was significantly decreased in the HGF-infused group, compared to the controls [$t(18) = 2.25$, $p < 0.048$; fig. 3a]. No significant effect of HGF on the number of white chamber entries was seen [$t(18) = 0.65$; fig. 3b]. The amount of conditioned fear stress-induced freezing behavior was significantly decreased in the HGF-infused group compared to the vehicle-treated group [$t(18) = 2.38$, $p < 0.036$; fig. 4]. No significant differences between the two groups were seen in general locomotor activity ($F_{2,35} = 1.30$; fig. 5).

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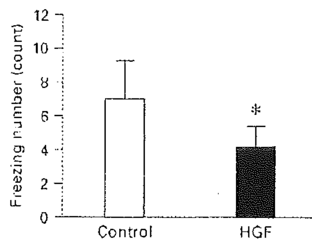

Fig. 4. Level of freezing induced by conditioned fear was significantly decreased in the HGF-infused group, compared to the controls. * $p < 0.05$ vs. vehicle- and HGF-infused group.

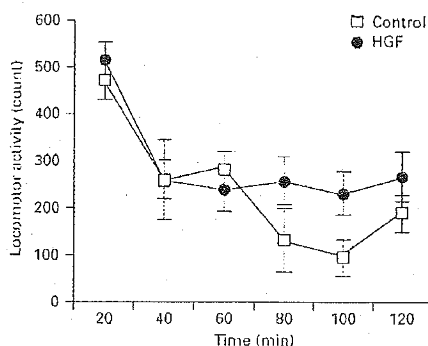

Fig. 5. No significant effect of HGF on general locomotor activity (number of photobeam breaks) was seen. Data are means ± SEM.

Discussion

This study provides the first evidence that HGF has an anxiolytic effect on the rat. HGF infusion into a lateral ventricle decreased anxiety as measured in the elevated plus maze and black and white box tests.

HGF was originally known as a cell mitogen and motogen, and has since been found to be a multifunctional growth factor with a variety of biological activities in numerous types of cells [17, 18]. The variety of biological functions attributed to HGF results from its interaction with its only known high-affinity transmembrane receptor, c-Met tyrosine kinase, present on target cells including central neurons [10, 19]. Coexpression of c-Met and HGF is oncogenic, and has been implicated in the progression of certain malignancies, in part, by decreasing tumor cell death and apoptosis [20, 21]. HGF and c-Met have been found to be present in specific subtypes of hippocampal neurons, cortex, septum, and cerebellum of both developing and adult mammalian brains [10, 12], but few reports exist concerning the biological activity of HGF in the CNS. A HGF-activating protease, HGF activator (HGFA), has recently been identified as a key enzyme that regulates the activity of HGF in vivo. HGFA appears to be associated with the cell surface. The HGFA antibody stained only astrocytes in the white matter in all the brain tissues. Expression of the mRNAs of HGF and HGFA was also seen in white matter astrocytes [22]. Recent studies have recognized effects of HGF on motor neuron survival, development and maturation, and on the function of cortical and hippocampal neurons in the developing brain [11, 12]. Tsuboi et al. [23] reported that consistent with the immunohistochemical data, a significantly higher concentration of HGF in Alzheimer's disease (AD) CSF was found as compared with controls. A significant correlation was also seen between CSF HGF levels and white matter high-signal foci determined on brain magnetic resonance imaging in AD patients. CSF HGF levels correspond with the white matter damage in AD brain [23].

Treatment with HGF induced an anxiolytic effect. But the mechanism of action of HGF has not been elucidated. The c-Met receptor has a heterodimeric protein which contains intracellular tyrosine kinase domains. Binding of HGF to c-Met might induce the anxiolytic effect [24]. HGF has the capacity to selectively direct thalamocortical projections into an intermediate target, the pallidum, and eventually to their final cortical destination. Mice with a targeted mutation of the gene encoding uPAR, a key component in HGF/SF activation and function, have decreased levels of HGF/SF and a 50% reduction in neocortical GABAergic interneurons at embryonic and perinatal ages. Mice of the uPAR –/– strain survive until adulthood, and behavior testing demonstrates that they have an increased anxiety state [14]. HGF may have a role in the mediation of anxiety.

In summary, this study reports that HGF infusion into the brain produced an anxiolytic effect in rats, as evaluated using the elevated plus maze, black and white box tests and conditioned fear test.

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（資料　３．）

平成１７年度

総括研究報告書

研究成果の刊行に関する一覧表よびその刊行物

厚生労働科学研究費補助金（こころの健康科学研究事業）
総括研究報告書

筋萎縮性側索硬化症に対する肝細胞増殖因子（HGF）を用いた挑戦的治療法の開発とその基盤研究

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研究要旨：本研究の目的は神経難病でも最も苛酷な筋萎縮性側索硬化症（ALS）に対して肝細胞増殖因子（HGF）を用いた挑戦的治療法を開発することとそれに関わる基盤研究を進めることにある。ALS の病因研究および治療研究には変異 Cu/Zn superoxide dismutase（SOD）遺伝子導入 ALS ラットが重要な役割を果している。私共はこの ALS ラットを用いて運動ニューロンに対し神経栄養因子作用を有するヒトリコンビナント Hepatocyte Growth Factor（HGF）の髄腔内持続投与で ALS に対する有効性を示してきた。今回 ALS への臨床応用を目指し ALS ラットにおける発症後からの HGF の髄腔内持続投与を行ない、対照 ALS ラットに較べ約 1.6 倍の延命効果を認めた。一方、抗 HGF 抗体を ALS ラットの髄腔内へ投与し HGF を中和させると病態を悪化させるので、HGF が ALS 病態の進行を抑制していることが示された。また、孤発性 ALS 患者の脊髄での残存運動ニューロンに HGF とその受容体 c-Met が発現していることが明らかにされ、外来性 HGF の投与が孤発性 ALS の病態進行抑制に有効である根拠が示された。この事実は今後の ALS への HGF 治療の臨床応用について大きなステップと考えられる。新規治療法開発の次なる段階としては、齧歯類に較べヒトにより近い霊長類における HGF 投与の安全試験が必要であり、ヒトの前臨床試験に向けて今後の開発研究の進展が期待される。

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A. 研究目的

筋萎縮性側索硬化症（ALS）は運動ニューロンの選択的な細胞死が惹起されて、全身の筋萎縮と脱力が進行する原因不明の難治性神経筋疾患である。しかも 2～3 年の経過で呼吸筋の麻痺をきたす極めて予後不良な疾患であるが、現状では有効な治療法がない。ALS の病因と病態の解明を行ない、それを基盤にした新規治療法の開発が世界的に切望されている。

わが国で発見された神経栄養因子である肝細胞増殖因子（Hepatocyte Growth Factor、以下 HGF）は、運動ニューロンに対する強力な保護作用が知られており、私

共は遺伝子工学的にALSマウスにおけるHGFの運動ニューロン死に対する抑制効果を明らかにしてきている。本研究の目的は、ALSの臨床応用を目指し、私共が開発した大型ALS動物モデルである変異Cu/Zn superoxide dismutase（SOD）導入ALSラットに対してHGF蛋白の髄腔内投与実験を行い、その有効性を確立することにある。

B. 研究方法

ALSの新規治療法の開発を目指しALSラットに対してヒトリコンビナントHGF蛋白の髄腔内持続投与による治療効果を明らかにし、ALSに対するHGFの臨床応用を目指して投与用量、投与時期および有効性の機序解明を行う。

その為に、以下の研究を行う。

1）新規治療法開発の基盤となる変異Cu/Zn SODの特性の検討と神経細胞死の機序解明

① Cu/Zn SODにはシステイン残基が4つありCys6とCys111はフリーのシステインで、Cys57とCys146はS-S結合をしている。Cys111は蛋白質の外側にあるGreek key loop内に存在し、ホモダイマーが向かい合った位置に存在するため、Cys111のSH基は特に反応性が高いと予想される。このCys111のSH基にSS交換反応で2-メルカプトエタノール（2-ME）を導入し、そのCu/Zn SOD蛋白質の酸化や安定性に及ぼす影響を検討した。

② 今まで神経組織特異的に発現する新規ユビキチンリガーゼNEDL1を同定し、このNEDL1は変異Cu/Zn SODとのみ強固な結合体を形成し、細胞内封入体に沈着することを確かめてきた。また、NEDL1はコロニー形成能の抑制とアポトーシス誘導能を有することを明らかにしてきた。今回は細胞死におけるNEDL1の関与を検討する目的で培養細胞にNEDL1およびp53の発現ベクター遺伝子導入して2週間にわたって形成されたcolony formation assayを行った。

2）臨床応用を目指したALSラットに対するHGFの髄腔内投与実験の完成とHGFの運動ニューロン死の抑制機序の解明

① HGFの臨床応用を目指してALSラットに対するHGFの髄腔内投与実験を行った。G93A Tgラットの髄腔内にヒトリコンビナントHGF（hr HGF）を浸透圧ポンプ（Alzet Model 2004）を用いて平均発症時期の115日齢からhr HGF 200μg/匹を持続投与して、死亡するまでの経過を観察した。内因性ラットHGFがALS病態に与える影響を検討する為に、内因性HGFが誘導されてくる週齢から4週間、ウサギポリクローナル抗ラットHGF特異抗体を5 μg/体重（g）髄腔内に持続投与し、発症日、死亡日を観察した。

② 今まで変異Cu/Zn SOD導入ALSマウスにて脊髄運動ニューロン変性に対するHGFの抑制効果を示してきたが、脳幹運動ニューロンに対する神経保護効果は不明であった。G93A TgマウスとHGF Tgマウスを交配してダブルトランスジェニックマウスを作製し、脳幹運動ニューロン（顔面神経核・舌下神経核）の変化とc-Metのチロシンリン酸を解析した。

③ 家族性ALS患者の脊髄残存前角神経細胞におけるHGF及びそのレセプターであるc-Metの発現を免疫組織学的に解析した。

孤発性 ALS 40 例、変異 Cu/Zn SOD を伴う家族性 ALS 2 家系 5 症例の各脊髄剖検材料を human HGF 抗体と human c-Met 抗体を用いて免疫染色を行った。

3) HGF を用いた将来的な ALS 治療としての再生医療や遺伝子治療の可能性

HGF を用いた再生医療の開発を目的に ALS ラットにおける内在性神経前駆細胞の HGF に対する反応を解析した。ALS Tg に対して hr HGF（総量 100 μg/匹）を 2 週間にわたって髄腔内に持続投与した後、BrdU で標識される新生細胞数を検討した。また、発症後の ALS ラットに対して上皮細胞成長因子（EGF）および線維芽細胞成長因子（FGF-2）を同時投与した群と単独投与群での内在性神経前駆細胞の賦活の有無を検討した。

（倫理面への配慮）

すべての遺伝子操作は本学 DNA 組換え実験指針に従い、また動物実験は同動物実験指針に従った上で動物愛護面に配慮しかつ利用動物数を極力減らすように務めた。

C 及び D. 研究結果及び考察

1）新規治療法開発の基盤となる変異 Cu/Zn SOD の特性の検討と神経細胞死の機序解明

① Cys111 にのみ 2-ME が結合するが、Cys6 には結合していないことが MALD-TOF-MASS 解析で確認された。2-ME がついた 2-ME-SOD は 75℃以上になると元の SOD に較べて熱に安定であることが示された。この結果から、2-ME 化した SOD は野生株の SOD よりも熱に安定で酸化されにくいことが明らかになった。以上の結果は、Cys111 の SH 基を 2-ME でマスクすると SOD を安定化させることが可能であることを示唆する。変異 SOD の Cys111 の SH を特異的に保護する薬剤の開発は家族性 ALS の治療につながる可能性がある。

② NEDL1 は総体的には変異 Cu/Zn SOD などのミスフォールド蛋白を認識する品質管理ユビキチンリガーゼである可能性が示唆されるが、長期的にはこの分解システムに破錠をきたし組織内凝集体の核になると考えられる。NEDL1 が細胞増殖あるいはアポトーシスにどのような機能を担うかを調べるため、培養細胞に NEDL1 を過剰発現させて colony formation assay を行った結果、アポトーシスが誘導された。そこで、細胞のアポトーシス誘導の重要な蛋白質である p53 の関与の有無について H1299 (p53-/-) と SH-SY5Y（野生型 p53）を用いて colony formation assay 法にて検討したところ、NEDL1 による細胞死の誘導は p53 を介した機構であることが明らかになった。NEDL1 が核内において p53 と結合し、その安定化を介してアポトーシスを誘導する機能を有していることは、家族性 ALS における神経細胞死の分子機序を考えるうえで、極めて重要な知見と考えられる。

2) 臨床応用を目指した ALS ラットに対する HGF の髄腔内投与実験の完成と HGF の運動ニューロン死の抑制機序の解明

① ALS ラットに対して hr HGF の髄腔内持続投与を ALS 発症期から行うことによって、平均死亡は HGF 投与群が 154.3 ±16.4 日、対照群が 143.25±17.0 日（p=0.02323）と HGF 投与群が対照群より有意

に遅延した。発症から死亡までの平均罹病期間が、HGF投与群が27.5±11.1日間、対照群が16.9±8.17日間と、HGF投与群では対照群の62.7%の増大を示し、発症期の投与によってもHGFがTgラットの罹病期間を大幅に延長させることが示された。発症時期からの投与により罹病期間延長効果が得られたことは、臨床への応用という点に関して注目すべき結果と考える。また、本ALSモデルラットにおいて運動ニューロン脱落とともに誘導されてくる内因性のラットHGFを抗HGF抗体の髄腔内投与によって中和すると、コントロール群に比して抗HGF抗体投与群では、より早期に発症する傾向（$p=0.1843$）、有意に速い進行（$p=0.0299$）、より早期の死亡が認められ（$p=0.0463$）、病態が悪化することが明らかとなった。このことから、HGFがALS様病態の進行を遅らせている重要な生理的抑制因子であることが示唆された。

② ダブルトランスジェニックマウスでは脳幹の運動ニューロン（顔面神経や舌下神経）の変性がALSマウスに較べて抑制されていることが示された。またc-Metのチロシンリン酸化によりHGFのシグナルを評価すると、HGFはALSの神経細胞においてより効率的にシグナル伝達することが明らかになった。

③ ALS患者脊髄組織の免疫染色の結果は、発症後約3年までの孤発性ALS脊髄残存神経細胞の一部には、HGFとその受容体c-Metが発現していた。しかし、それ以降の臨床経過の長い孤発性ALSでは経過と共にHGF-c-Metシステムがup-regulateしている残存神経細胞数は激減していった。これらの免疫染色の結果はALSでの大部分の残存神経細胞はHGF-c-Metシステムが破錠するために細胞死に至るが、一部にはHGF-c-Metシステムを発現して存在する可能性を保っていることが判明した。

以上より、外来性HGFの供給は生理的なHGFの病態進行抑制作用を強化するという点でヒトALSに対する理論的かつ有力な新規治療法になり得ると考えられる。

3）HGFを用いた将来的なALS治療としての再生医療や遺伝子治療の可能性

これまでに、ALSラットモデルの脊髄では発症期以前からグリア新生（gliogenesis）の亢進が認められるとともに、末期には未分化神経前駆細胞も増殖することを報告してきた。HGF投与によって病期の進行に伴い、BrdU陽性細胞数が増加し、ALS病態下で増加しつつある新生細胞をさらにHGFが増加させることが示された。さらにはEGFとFGF-2を同時に髄腔内持続投与することによって発症後のALSラットモデルの脊髄での新生細胞をさらに増加させられることが示された。今後はより効果的な内在性神経前駆細胞活性化のために至適投与時期と投与量の検討が必要と考えられる。

E. 結論

本研究の目的は神経難病でも最も苛酷な筋萎縮性側索硬化症（ALS）に対して肝細胞増殖因子（HGF）を用いた挑戦的治療法を開発することとそれに関わる基盤研究を進めることにある。ALSの病因として最も重要視されている変異Cu/Zn SODによる選択的運動ニューロン死のメカニズムはまだ十分明らかにされていないが、変異Cu/Zn SOD導入によるALS Tgラットは病

因・治療研究に極めて有用なモデルである。

今回の治療実験で ALS ラットにおける発症後からの HGF の髄腔内持続投与にて明らかな延命効果が認められた。この事実は今後の ALS の HGF 治療の臨床応用について大きなステップと考えられる。また、抗 HGF 抗体を ALS ラットの髄腔内へ投与し HGF を中和させると病態を悪化させるので、HGF が ALS 病態の進行を抑制していることが示された。また、孤発性 ALS 患者の脊髄での残存運動ニューロンに HGF とその受容体 c-Met が発現していることが明らかにされ、外来性 HGF の投与が ALS の病態進行抑制に有効である根拠が示された。新規治療法開発の次なる段階としてはこれらの結果をふまえ、齧歯類に較べヒトにより近い霊長類における HGF 投与の安全試験が必要であり、ヒトの前臨床試験に向けて今後の研究進展が期待される。

F. 健康危険情報

特になし

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許登録

ラットを用いたALSモデル（出願済）

2. 実用新案登録

なし

3. その他

なし

Journal of Cerebral Blood Flow & Metabolism (2006), 1–13
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www.jcbfm.com

Novel therapeutic strategy for stroke in rats by bone marrow stromal cells and *ex vivo* HGF gene transfer with HSV-1 vector

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Occlusive cerebrovascular disease leads to brain ischemia that causes neurological deficits. Here we introduce a new strategy combining mesenchymal stromal cells (MSCs) and *ex vivo* hepatocyte growth factor (HGF) gene transferring with a multimutated herpes simplex virus type-1 vector in a rat transient middle cerebral artery occlusion (MCAO) model. Gene-transferred MSCs were intracerebrally transplanted into the rats' ischemic brains at 2 h (superacute) or 24 h (acute) after MCAO. Behavioral tests showed significant improvement of neurological deficits in the HGF-transferred MSCs (MSC-HGF)-treated group compared with the phosphate-buffered saline (PBS)-treated and MSCs-only-treated group. The significant difference of infarction areas on day 3 was detected only between the MSC-HGF group and the PBS group with the superacute treatment, but was detected among each group on day 14 with both transplantations. After the superacute transplantation, we detected abundant expression of HGF protein in the ischemic brain of the MSC-HGF group compared with others on day 1 after treatment, and it was maintained for at least 2 weeks. Furthermore, we determined that the increased expression of HGF was derived from the transferred *HGF* gene in gene-modified MSCs. The percentage of apoptosis-positive cells in the ischemic boundary zone (IBZ) was significantly decreased, while that of remaining neurons in the cortex of the IBZ was significantly increased in the MSC-HGF group compared with others. The present study shows that combined therapy is more therapeutically efficient than MSC cell therapy alone, and it may extend the therapeutic time window from superacute to acute phase.
Journal of Cerebral Blood Flow & Metabolism advance online publication, 18 January 2006; doi:10.1038/sj.jcbfm.9600273

Keywords: gene transfer; hepatocyte growth factor; herpes simplex virus; intracerebral transplantation; mesenchymal stromal cell; transient cerebral ischemia

Introduction

Occlusive cerebrovascular disease often causes global ischemia of the brain and results in neuropathological changes. Several methods have been proposed to augment brain reorganization, including the stimulation of endogenous processes through pharmacologic or molecular manipulation, gene therapy, behavioral and rehabilitation strategies, and the provision of new substrates for recovery through cell therapy.

Bone marrow contains the precursors of nonhematopoietic tissues that are referred to as mesenchymal stem cells or marrow stromal cells (MSCs) (Friedenstein *et al*, 1978). Marrow stromal cells are characterized by the ability to self-renew in a number of nonhematopoietic tissues, and by their

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This work was supported by Grants-in-Aid for Scientific Research (B) (14370448) and (C) (12671353), and by a Grant-in-Aid for Exploratory Research (14657350) from the Japanese Ministry of Education, Science and Culture, Japan to Shin-Ichi Miyatake, MD, PhD. Additional support was provided in the form of a grant from the Special Assistance for Promoting the Advancement of the Education & Research of the Private University, Promotion and Mutual Aid Corporation for Private Schools of Japan and the Science Research Promotion Fund to Shin-Ichi Miyatake, MD, PhD, and by the High-Tech Research Program of Osaka Medical College. This work was also supported in part by Grants-in-Aid 17790989 from the Ministry of Education, Science and Culture, Japan to Naosuke Nonoguchi, MD.
Received 22 August 2005; revised 29 November 2005; accepted 5 December 2005

multipotentiality for differentiation into various tissues, such as fibroblasts, bone, muscle, and cartilage (Caplan and Bruder, 2001; Phinney, 2002). Additionally, they share some characteristics of neurons and astrocytes when cultured *in vitro* (Kim *et al*, 2002) or after being implanted into the central nervous system *in vivo* (Chopp *et al*, 2000; Nakano *et al*, 2001; Li *et al*, 2001, 2002; Chen *et al*, 2002*a*, *b*). Marrow stromal cells can also secrete growth factors and cytokines into the soluble stromal and neurochemicals into the brain (Li *et al*, 2002; Chen *et al*, 2002*a*, *b*), cross the blood–brain barrier (BBB) and migrate throughout the brain preferentially to areas that have suffered damage (Chen *et al*, 2000; Li *et al*, 2000, 2001; Damme *et al*, 2002). Many previous researchers have reported on mesenchymal stromal cell (MSC) transplantation as a source for autoplastic therapies and improvement in functional recovery after stroke (Chen *et al*, 2000; Li *et al*, 2000, 2001, 2002; Rempe and Kent, 2002; Kurozumi *et al*, 2004).

Hepatocyte growth factor (HGF) is a disulfide-linked heterodimeric protein that was initially purified and cloned as a potent mitogen for hepatocytes and a natural ligand for the c-met proto-oncogene product (Nakamura *et al*, 1984; Matsumoto and Nakamura, 1996). Subsequently, several functions have been ascribed to HGF, including antiapoptosis, angiogenesis, motogenesis, morphogenesis, hematopoiesis, tissue regeneration in a variety of organs, and the enhancement of neurite outgrowth (Matsumoto and Nakamura, 1997; Hayashi *et al*, 2001; Sun *et al*, 2002*a*, *b*; Jin *et al*, 2003). It has also been reported that HGF administration could inhibit the BBB destruction, decrease brain edema, and provide a neuroprotective effect after brain ischemia (Miyazawa *et al*, 1998; Hayashi *et al*, 2001; Shimamura *et al*, 2004).

Recent experimental studies suggest the possibility that gene transduction into MSCs could enhance their existing therapeutic potential (Chen *et al*, 2000; Kurozumi *et al*, 2004). Here, we evaluate the efficiency and effects of gene transduction into MSCs using a replication-incompetent herpes simplex virus type-1 (HSV1764/4-/pR19) vector disabled by the deletion of three critical genes for viral replication encoding infected cell polypeptide (ICP)4, ICP34.5, and virion protein (VP16) (vmw65). This vector contains HSV latency-associated transcript (LAT) promoter and two kinds of enhancer elements: cytomegalovirus (CMV) enhancer and Woodchuck posttranscriptional regulatory elements (WPRE). The availability of this vector has already been examined in the nervous system (Coffin *et al*, 1998; Palmer *et al*, 2000; Lilley *et al*, 2001).

In the present study, we intracerebrally transplanted MSCs in which a gene of interest was transferred with this HSV-1 vector *ex vivo* into a rat transient middle cerebral artery occlusion (MCAO) model under superacute and acute therapeutic time phase, and investigated whether such combined therapy could improve the effects of ischemia.

Materials and methods

Donor Cell Preparation

Marrow stromal cells of adult Wistar rats were prepared following the method described by Azizi *et al* (1998). In brief, the marrow of rat tibias and femurs was extruded with 10 mL of alpha-MEM (Sigma Chemical Co., St Louis, MO, USA) and cultured in the same medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, and antibiotic–antimycotic 1 mL/100 mL (GIBCO Invitrogen, Carlsbad, CA, USA) at 37°C, 98% humidity and 5% CO_2. After 48 h, the nonadherent cells were removed by replacing the medium, and the adherent cells were continuously subcultured as MSCs. The fifth to seventh passages were used for the following experiments.

HSV1764/-4/pR19-Hepatocyte Growth Factor Virus and Propagation

One of the authors of the current study (Coffin) constructed the prototype HSV1764/4/pR19GFP virus and has previously described this vector's characteristics (Palmer *et al*, 2000; Lilley *et al*, 2001), which are also described briefly in the Introduction. In the present study, the green fluorescent protein (*GFP*) gene was replaced with a full-length rat HGF complementary DNA (cDNA) tagged with the KT3 (SV (simian virus)40 large, T antigen) epitope (ratHGFKT3) (Sun *et al*, 2002*b*), and the authenticity of this vector (pR19ratHGFKT3WPRE) was confirmed by sequence analysis. Homologous recombination was performed in M49 cells by cotransfection of plasmid pR19ratHGFKT3WPRE DNA and HSV1764/-4/pR19GFP viral DNA. White plaques were selected and purified three times, and replication-incompetent viruses were propagated as described previously (Palmer *et al*, 2000). We ultimately obtained the HSV1764/-4/pR19-HGF virus (HSV-HGF) with a titer of 2×10^{8} pfu/mL for use in the present experiments.

Ex Vivo Gene Delivery to MSCs

The cultured MSCs from the fifth to seventh passages were infected with the virus suspension by incubation for 1 h. After infection, the virus suspension was changed to normal culture medium for MSCs and continuously cultured for the subsequent 24 h before transplantation.

Our previous experiments show that the transduction efficiency of the *GFP* gene into the MSCs with our HSV-1 vector is more than 50% even with a multiplicity of infection (MOI) of 5. Here we set the MOI at 5 for the desired gene transfer to MSCs *ex vivo*.

Hepatocyte Growth Factor Detection with Enzyme-Linked Immunosorbent Assay (ELISA) *In Vitro*

We prepared 1.6×10^5 MSCs in each well of a six-well dish. The MSCs were transferred with *HGF* gene by infection with HSV-HGF at MOIs of 0, 0.1, 1, 5, and 10. At 1 h after infection, the infected MSCs were successively incubated with normal culture medium for another 24 h. The culture supernatant and cells were then individually collected through centrifugation. The HGF protein concentrations in MSC culture supernatant and in MSC extracts prepared using 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride (PMSF) (Wako, Osaka, Japan), 2 μg/ml antipain (Peptide Institute Inc., Osaka, Japan), 2 μg/ml leupeptin (Peptide Institute), and 2 μg/ml pepstatin (Peptide Institute) were determined by ELISA using an anti-rat HGF polyclonal antibody (Tokushu Meneki, Tokyo, Japan) as described (Sun *et al*, 2002*b*).

Transient Middle Cerebral Artery Occlusion Animal Model

Experiments were performed on 8-week-old male Wistar rats weighing 250 to 280 g. We induced transient MCAO using the previously described method of intraluminal vascular occlusion (Longa *et al*, 1988). In brief, a length (18.5 to 19.0 mm, determined according to the animal's weight) of 4-0 surgical nylon suture was gently advanced from the external carotid artery into the lumen of the internal carotid artery until it reached the proximal segment of the anterior cerebral artery. After 2 h of MCAO the animals were reanesthetized, and reperfusion was achieved by withdrawing the nylon suture.

The rats were subjected to transient MCAO for 2 h to produce a consistent and reproducible ischemic lesion in the unilateral striatum and cortex.

Intracerebral Transplantation of MSCs

At 2 or 24 h after the onset of MCAO (i.e., on reperfusion), the animals were placed in a stereotactic head holder (model 900, David Kopf Instruments, Tujunga, CA, USA) under inhalation anesthesia. MSCs were intracerebrally transplanted by inserting a 26-gauge needle with a Hamilton syringe into the right striatum (anteroposterior (AP) = 0 mm; lateral to midline (ML) = 2.0 mm; vertical to dura (DV) = 4.5 mm) from bregma, based on the atlas given by Paxinos *et al* (1985). There were 1×10^6 cells in total 10-μl fluid volumes that transplanted into each animal over a 10-min period. No immunosuppressive drugs were used in any animal.

Experimental Groups

In this study, there were seven experimental groups: groups 1 and 5 were treated with phosphate-buffered saline (PBS); groups 2 and 6 were treated with untreated MSCs only; group 3 was treated with the GFP-transferred MSCs (MSC-GFP); and groups 4 and 7 were treated with *HGF* gene-transferred MSCs (MSC-HGF).

Groups 1 to 4 were treated 2 h after MCAO (superacute phase) and groups 5 to 7 were treated 24 h after MCAO (acute phase).

Behavioral Testing

The rats of groups 1 to 4 ($n = 6$) were subjected to a modified neurological severity score (mNSS) test (Schallert *et al*, 1997) to evaluate neurological function before MCAO, at 2 h after MCAO, and at 1, 4, 7, 14, 21, 28, and 35 days after MCAO. The rats of groups 5 to 7 ($n = 6$) were subjected to mNSS before MCAO and at 0, 1, 4, 7, and 14 days after MCAO. These tests are battery of motor, sensory, reflex, and balance tests, which are similar to the contralateral neglect tests in humans. The higher the score, the more severe the neurological deficit (Chen *et al*, 2001).

Infarction Volume

We stained the brains of groups 1, 2, and 4 ($n = 6$) and groups 5 to 7 ($n = 5$) with 2,3,5-triphenyltetrazolium chloride (TTC) (Wako Pure Chemical Industries, Osaka, Japan) to detect the infarction volume of each group at 3 and 14 days after treatment. Briefly, the rats' brains were removed and cut into seven equally spaced (2 mm) coronal sections. These sections were immersed in a 2% solution of TTC at 37°C for 20 mins to reveal the infarcted areas. This procedure is known to reliably mark ischemic damage even at 14 days after MCAO (Bederson *et al*, 1986; Kurozumi *et al*, 2005).

The disposition of the ischemic area was evaluated by calculating the hemispheric lesion area using imaging software (Scion Image, version Beta 4.0.2; Scion Corp., Frederick, MD, USA). To avoid overestimation of the infarct volume, the corrected infarct volume (CIV) was calculated as $CIV = [LT - (RT - RI)] \times d$, where LT is the area of the left hemisphere, RT is the area of the right hemisphere, RI is the infarcted area, and d is the slice thickness (2 mm) (Raymond *et al*, 1990). Relative infarct volumes are expressed as a percentage of contralateral hemispheric volume.

Terminal Deoxynucleotidyltransferase (dUTP) Nick End-Labeling (TUNEL) Staining and Immunohistochemical Assessment

Sample Preparation: At different time points, rats of groups 1, 2, and 4 were reanesthetized and transcardially perfused with saline, followed by 4% paraformaldehyde in PBS. The brain tissues were cut into seven equally spaced coronal blocks. The tissues were processed and 10-μm cryosections were cut.

Immunohistochemical Staining: We can detect three kinds of HGF in this study: the endogenous HGF secreted by the rat ischemic brain tissue after stoke (en-HGF), the exogenous HGF secreted by the transplanted MSCs (ex-HGF-1), and the exogenous HGF delivered from the

HSV-HGF (ex-HGF-2). For the immunohistochemical staining of HGF, the whole rats' brain sections of groups 1, 2, and 4 were prepared on days 2 and 14 after treatment. Rabbit anti-rat HGF primary antibody (prepared by some of the authors of this article, and belonging to the Division of Molecular Regenerative Medicine, Osaka University Graduate School of Medicine, Japan) was used to detect the three kinds of HGF (mixed); a KT3 primary monoclonal antibody (1:1000) (Covance Research Products, Berkeley, CA, USA) was used to detect the ex-HGF-2; a biotinylated universal secondary antibody (VECSTAIN Elite ABC Kit, PK-6200, Vector Laboratories, Burlingame, CA, USA) and a goat anti-rabbit IgG affinity-purified rhodamine-conjugated secondary antibody (1:200) (Chemicon International, Temecula, CA, USA) were also used here. Reaction products were visualized with the VECSTAIN Elite ABC Kit (PK-6200) and a DAB Substrate Kit (Vector Laboratories, Burlingame, CA, USA). To detect the donor MSCs, bisbenzimide (Hoechst 33258; Polysciences, Eppelheim, Germany) was used to fluorescently label cell nuclei *in vitro*. Some sections were counterstained with hematoxylin and observed under a normal light microscope (VB-S20 Multiviewer System, Keyence, Osaka, Japan and Microphot-FXA, Nikon Corp., Tokyo, Japan), and some were directly observed by a fluorescence microscope (BX-50-34-FLAD1, Olympus). The donor MSCs could be detected under ultraviolet (UV) light with blue fluorescence as marked by Hoechst 33258.

To visualize the remaining neurons in the cortex of the ischemic boundary zone (IBZ) of groups 1, 2, and 4 ($n=3$), 7 days after treatment, microtubule-associated protein 2 (MAP-2) was used as the first antibody (1:500) (Chemicon International Inc., CA, USA). Negative control slides for each animal received identical preparation for immunohistochemical staining, except that primary antibodies were omitted.

Terminal Deoxynucleotidyltransferase Nick End-Labeling Staining: At 7 days after treatment, coronal cryosections (10-μm thick) of each rat of groups 1, 2, and 4 ($n=3$) were stained by the TUNEL method for *in situ* apoptosis detection (ApopTag kit, Chemicon International, USA). Specifically, after postfix slides were incubated in a mixture containing terminal deoxynucleotidyl transferase and anti-digoxigenin-rhodamine (Red). Then, they were counterstained with bisbenzimide (Hoechst 33258), which stains blue for each nucleus. The total numbers of TUNEL-positive cells and Hoechst counter-staining positive cells were individually counted in 2 slides from each brain, with each slide containing five random fields from the IBZ, under an $\times$ 20 objective of the fluorescence microscope system (BX-50-34-FLAD1, Olympus), using a 3-CCD color video camera (Keyence VB-7010, Keyence, Osaka, Japan).

Statistical Analysis

Data are presented as means$\pm$standard deviations (s.d.). Data from the behavior test (mNSS) were evaluated with repeated-measures analysis of variance (ANOVA), with subsequent Fisher's protected least significant difference (PLSD) test. StatView 5.0 software (SAS Institute, Cary, NC, USA) performing the Student's *t*-test was used to test the CIV data and the difference in means of percentage of the apoptosis-positive cells and the remaining neurons. A difference with a probability value of $P \leq 0.05$ was considered to be statistically significant.

Results

Quantification of Hepatocyte Growth Factor Analysis with Enzyme-Linked Immunosorbent Assay *In Vitro*

As a result, the HGF concentration was approximately 15 times higher in the culture supernatant than in the cell extract at the same MOI, and its increase was correlated with an increase in MOI. Although normal MSCs can produce HGF protein at 0.4 ng/1.6 $\times$ 10^5 cells/24 h, after the MSCs were infected with HSV-HGF at an MOI of 5, they were found to produce HGF protein at 2.4 ng/1.6 $\times$ 10^5 cells/24 h (Figure 1).

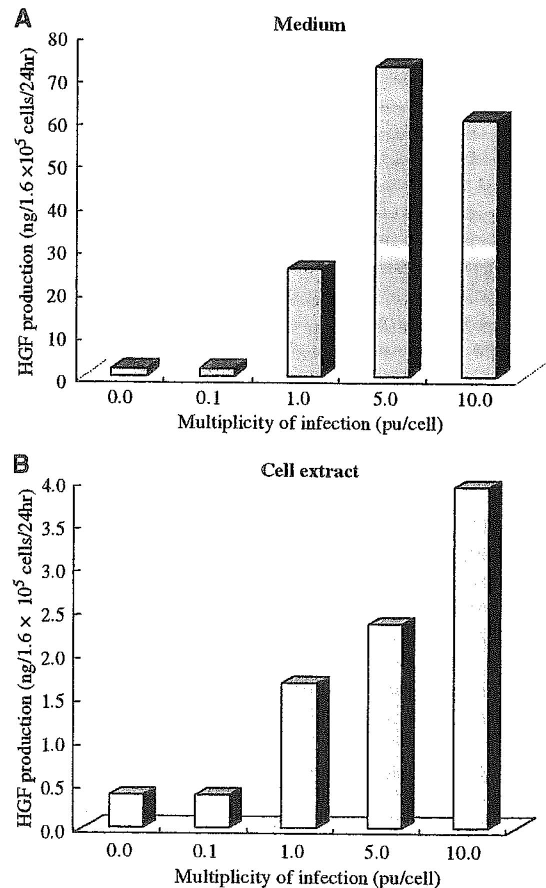

Figure 1 Enzyme-linked immunosorbent assay to determine HGF concentration *in vitro*. Hepatocyte growth factor concentrations were detected in MSC culture supernatant (**A**) and in MSC cell extract. (**B**) After 24 h, 1.6 $\times$ 10^5 MSCs were transfected with HSV-HGF at MOIs of 0, 0.1, 1, 5, and 10.

Neurological Outcome

No significant difference in neurological function was detected among all the groups just before cell transplantation. Significant differences of functional recovery were found in group 1 individually compared with group 2 (days 14 to 35, $P<0.05$), with group 3 (days 21 to 35, $P<0.05$), and with group 4 (days 4 and 7, $P<0.05$; days 14 to 35, $P<0.01$) during the observation periods after the superacute transplantation (Figure 2A), and in group 5 individually compared with group 6 (day 14, $P<0.05$), with group 7 (day 7, $P<0.01$ and day 14, $P<0.01$) after the acute transplantation (Figure 2B). Interestingly, we observed significant differences of functional recovery on day 14 among all the superacute treated groups including the MSC-GFP group, which served as a control for *ex vivo* nontherapeutic gene transduction ($P<0.05$). Exceptionally, there was no significant difference only between the MSC-only and the MSC-GFP groups at that time point (Figure 2A). We also found significant neurological recovery on day 14 in the combined therapy group treated even in the acute phase, compared with the MSC-only group treated in the superacute phase (Figure 2C). Also, significant difference of functional recovery on day 14 was found among the groups treated in the acute phase (Figure 2B).

Quantitative Analysis of Infarct Volume

We compared the infarction areas in coronal sections of groups 1, 2, and 4 on day 3 (Figure 3A) and day 14 (Figure 3B), and compared those of groups 5 to 7 on the same time points by TTC staining, and expressed lesion volume as a percentage of contralateral hemispheric volume. At 3 days after treatment, significant difference of %CIV was only detected in the MSC-HGF group compared with the PBS group ($34.52\% \pm 3.44\%$ versus $41.83\% \pm 6.25\%$, $P<0.05$), both of which were treated in the superacute phase (Figure 3C). However, on day 3 there was no significant difference of %CIV among any group that was treated in the acute phase (Figure 3C), while on day 14 there were significant reductions of %CIV in the rats of the MSC-HGF group compared with not only the PBS group but also the MSC-only group treated in the both therapeutic phases (Figure 3D). Also on day 14, the rats treated with MSC-only showed significant reduction in %CIV compared with the PBS group that was treated in the superacute phase (Figure 3D).

Hepatocyte Growth Factor and herpes simplex virus type Gene-Transferred Hepatocyte Growth Factor Detection *In Vivo*

The macrographs presented in Figure 4 showed that mixed HGF protein was diffusely overexpressed in almost the whole ipsilateral brain in the MSC-HGF group compared with other groups, not only on day 2 (column A) but throughout at least the first 2 weeks (column C) after treatment. The microphotographs presented in column B of Figure 4 showed that high HGF expression in the MSC-HGF group could be detected in both the ipsilateral cortex and the ipsilateral basal ganglia at 2 days after treatment. Nevertheless, almost no HGF expression could be detected on the contralateral hemisphere in any treatment group (Figure 4).

Fluorescent staining of groups 1, 2, and 4 on day 14 (Figure 5, column C) also showed higher mixed HGF expression in the MSC-HGF group than that of the other groups. Also, we could detect donor MSCs with blue fluorescence expression by direct observation under UV light (Figure 5, column B). We could identify the HGF expression with red fluorescence in both the transplanted cells and the intercellular space in the transplantation area.

Furthermore, we detected ex-HGF-2 expression, which was transferred from HSV-HGF by anti-KT3 staining (Figures 6D to 6F) of the implantation area. As a result, we had identified ex-HGF-2 expression both in the *HGF* gene-transferred MSCs (arrows in Figure 6G) and in the intercellular space of the transplantation area (arrowheads in Figure 6G) only in the MSC-HGF group (Figure 6F) even 14 days after transplantation. Additionally, we confirmed that MSC itself can also secrete HGF *in vivo* (Figure 6B).

Antiapoptosis

Using TUNEL staining (Figure 7, columns B and C), apoptotic cells with red fluorescence were counted in the IBZ 7 days after treatment, while cells were counted in the same area with blue fluorescence by Hoechst 33258 nuclei marking. In this area we could not detect transferred MSCs; therefore, counterstained cells seemed to be host-derived. The percentage of apoptotic host cells was significantly decreased in the MSC-HGF group ($4.92\% \pm 2.15\%$) compared with the PBS group ($22.12\% \pm 4.28\%$, $P<0.01$) and MSC-only group ($10.73\% \pm 5.64\%$, $P<0.01$). However, there was also significant decrease of apoptotic cells between the MSC-only group ($10.73\% \pm 5.64\%$) and the PBS group ($22.12\% \pm 4.28\%$, $P<0.01$) (Figure 7C).

Neuroprotection

Immunohistochemical staining revealed the remaining neurons of the host with MAP-2 neuronal marker 7 days after treatment (Figure 8A). The percentage of remaining neurons in the cortex of IBZ significantly increased in the MSC-HGF group ($20.73\% \pm 2.38\%$) compared with the PBS group ($7.75\% \pm 1.58\%$, $P<0.01$) and the MSC-only group ($12.13\% \pm 3.05\%$, $P<0.01$). Also, the significant

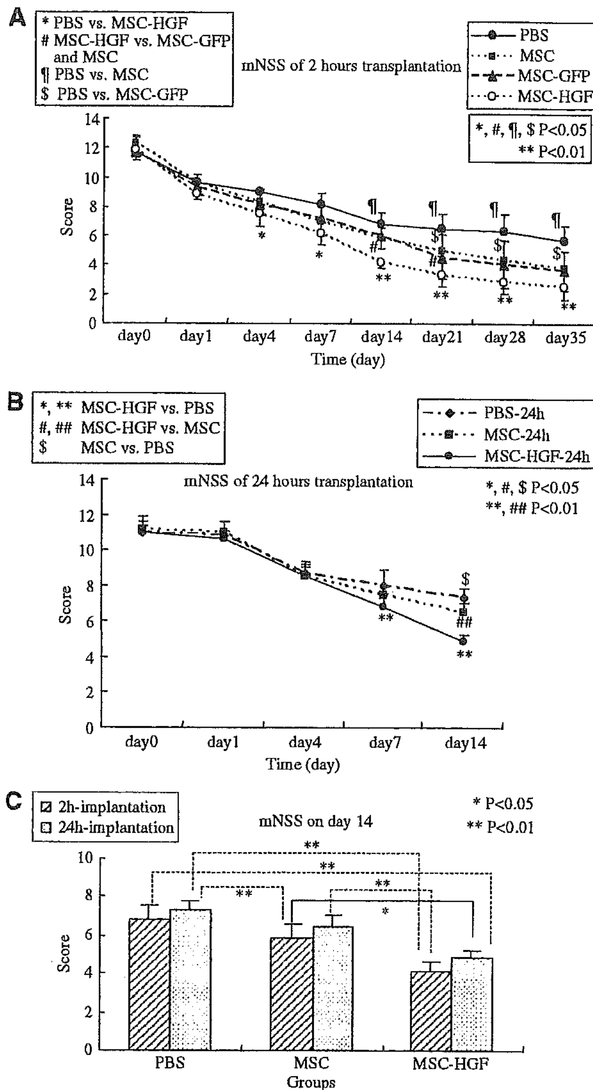

Figure 2 Behavioral functional test (mNSS) before and after MCAO. Groups 1 and 5: treated with PBS; groups 2 and 6: treated with MSC-only; group 3: treated with MSC-GFP; groups 4 and 7: treated with MSC-HGF ($n = 6$ per group). The rats of groups 2 to 7 received 1.0×10^6 cells via intracerebral transplantion in 10 μl PBS. (**A**) Groups 2 to 4 received transplantation 2 h after MCAO (superacute phase); (**B**) groups 4 to 7 received transplantation 24 h after MCAO (acute phase). (**C**) Lists the mNSS on day 14 of groups 1, 2, 4, 5, 6, and 7, showing that the significant neurological recovery among 6 groups while under the comparing condition is only the different therapeutic time phase. Significant functional recovery was detected in the MSC-HGF group compared with the other groups. Data are presented as means±s.d.

increase of remaining neurons was found in the MSC-only group (12.13%±3.05%), in comparison with the PBS group (7.75%±1.58%, $P<0.01$) (Figure 8B).

Discussion

Brain ischemia initiates a cascade of events that produces neuronal death and leads to neurological deficits. To prevent brain injury after ischemia, some studies have focused on cell therapies by using embryonic stem cell. But ethical and logistical problems make it unlikely that such therapy could serve as a source of material for therapeutic transplants. Recently, MSC transplantation was reported as a source of autoplastic therapies which not only improve functional recovery after stroke but also have a low risk of tumorigenesis and do not provoke immune reactions (McIntosh and Bartholomew, 2000; Li *et al*, 2002). In the present study, rats of the MSC-only and MSC-HGF groups also showed more significant neurological functional recovery than those of the PBS group.

It is well known that the efficiency of gene transduction to such MSC populations is low, even with virus vectors such as an adenovirus (Ad) (Conget and Minguell, 2000). To date, Kurozumi *et al* (2004) have reported the relatively high efficiency of gene transduction using fiber mutant Ad vector, but the peak level of expression was transient because the Ad vector would not integrate the gene of interest into the genome of the host cells. Lentivirus could express a high efficiency of gene transduction into MSC, but its biosafety remains uncertain because of its origin, the human immunodeficiency virus (Trono, 2000). Retroviruses, which have the ability to integrate the gene of interest into the chromosomes of the host cells, also show a relatively high efficiency of gene transduction to MSC. However, a note of warning was stressed against the potential rise of a neoplasm with a retrovirus-based vector (Pages and Bru, 2004).

In the present study, by the *in vitro* HGF ELISA data and histological detection, we showed that our HSV-1 vector had successfully transferred the gene of interest to the MSC population with high efficiency *in vitro*, and gene-transferred MSCs had successfully functioned *in vivo* to express and maintain a high level of the gene of interest. We confirmed that the increased HGF expression on day 14 was primarily due to the ex-HGF-2 expression that was proven by anti-KT3 staining, as the HSV-1 vector-transferred HGF cDNA was tagged with KT3 epitope. Also, such ex-HGF-2 protein was produced within the *HGF* gene-transferred MSCs and secreted in the intercellular space diffusely in the combined therapy group.

Furthermore, there were no significant differences in functional recovery between the MSC-only group and the MSC-GFP group during the whole detection time course. Also, no obvious difference of apoptosis and the dividing ability was observed between naive MSCs and the *HGF* gene-transferred MSCs in the current study in the first 2 weeks after transplantation (data not shown). It may indicate that gene transfer with HSV-1 vector *ex vivo* would

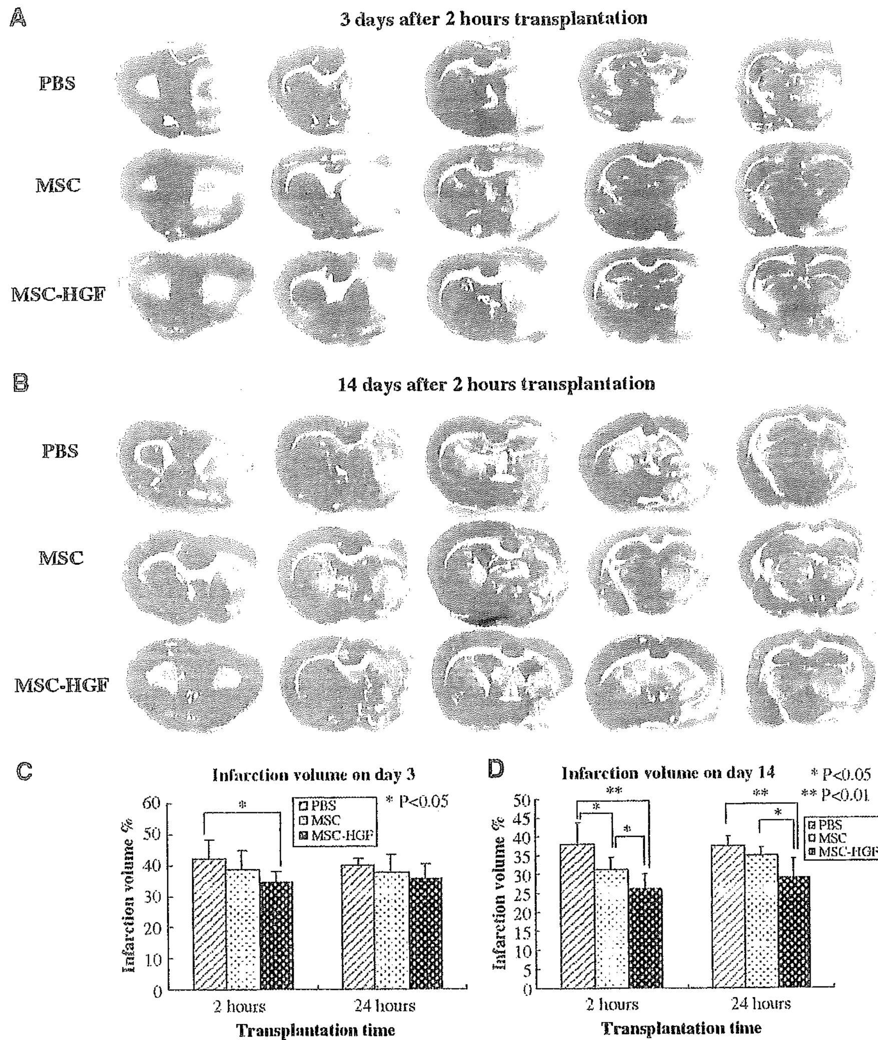

Figure 3 Infarction volume detected by TTC staining. (**A**, **B**) Reduction of infarction areas on days 3 and 14 of groups 1, 2, and 4, which received transplantation 2 h after MCAO occurred: coronal sections stained with TTC. The red region shows intact area; white region shows infarction area. (**C**, **D**) Individually presents the quantification of % CIV in the hemispheric lesion area on days 3 and 14, while being treated at 2 and 24 h after ischemia occurred. Data are presented as means±s.d. ($P < 0.05$; < 0.01). $n = 6$ for groups 1, 2, 4, and $n = 5$ for groups 5 to 7 at each time point.

not influence the survival and dividing abilities and the therapeutic efficiency of MSCs after transplantation.

So far, to reduce the disability resulting from stroke, some studies have focused on the development of neuroprotective agents such as brain-derived neurotrophic factor, the fibroblast growth factor that effectively prevents delayed neuronal death after transient brain ischemia (Kurozumi *et al*, 2004; Watanabe *et al*, 2004). Recently, overexpression of HGF that can improve the neurological sequalae by neuroprotection, reduce the infarct volume, and the likelihood of brain edema after stroke was reported (Miyazawa *et al*, 1998; Tsuzuki *et al*, 2000; Hayashi *et al*, 2001; Shimamura *et al*, 2004). It suggested that HGF should be one of the most potent growth factors for treating brain ischemia.

To detect the therapeutic efficiency of combined therapy, we tried to treat brain ischemia in the superacute and acute therapeutic phases. Both of them showed significant improvement of neurological deficits compared with MSC-only cell therapy. We got the same result as that Shimamura *et al* (2004) had reported, that HGF had the therapeutic efficiency of reducing the infarction volume after transient MCAO. We also found on day 14 that the MSC-only treated group could significantly reduce the infarction volume under the superacute treatment compared with the PBS-treated group, but not