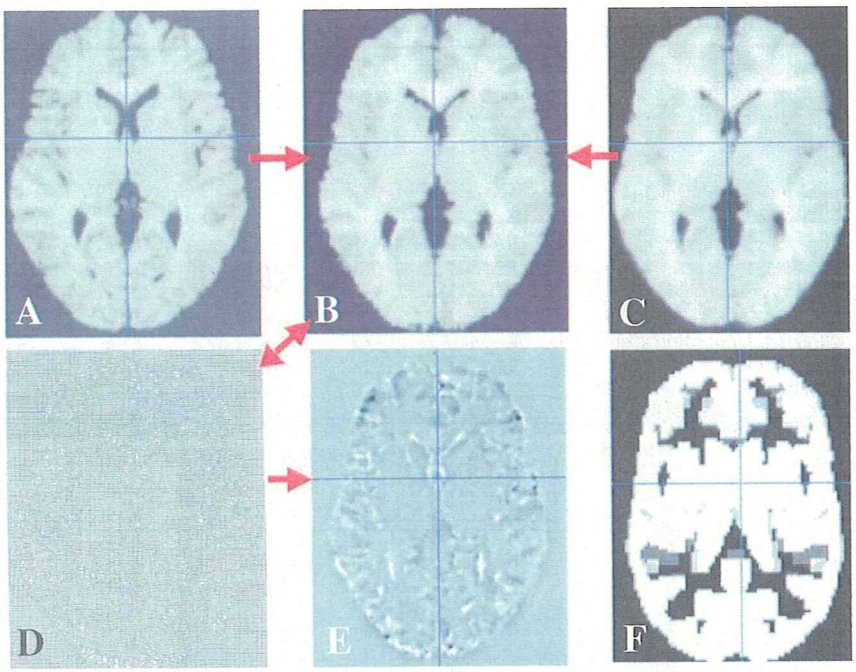

Fig. 1 Steps of analysis for tensor-based morphometry. An example is shown for a single subject in one axial slice. The single object brain (**A**) has been corrected for orientation and overall size to the template brain (**C**). Non-linear spatial normalization removes most of the anatomical differences between the two brains by introducing local deformations to the object brain, which then (**B**) looks as similar as possible to the template. Image (**D**) shows the deformations applied to the object brain by a deformed grid. Statistical analysis can be done univariate using the local Jacobian determinant as a derivative of the field (**E**). An explicit mask image (**F**) was used to explore morphology in the grey matter and CSF space.

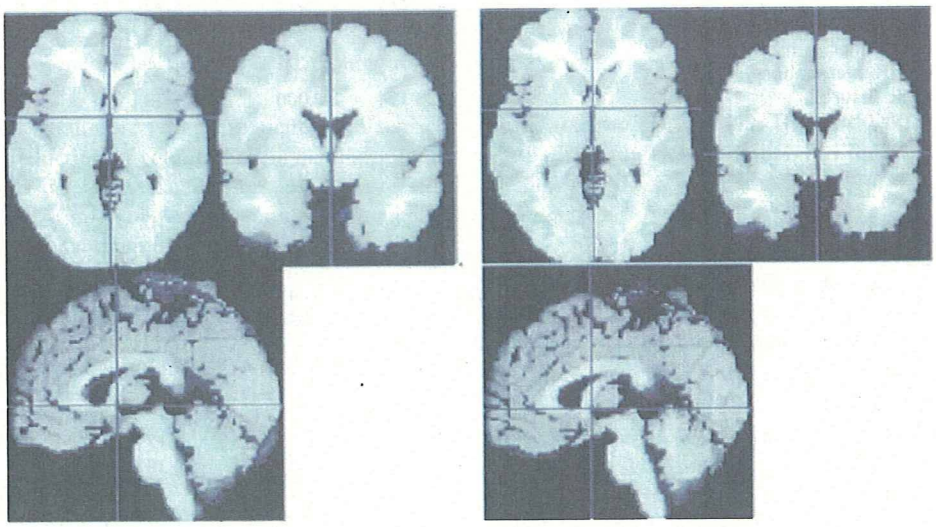

Fig. 2 Mean images after high dimensional warping control subjects and schizophrenics. *Left*: The mean image of warped MR images obtained from 76 controls. Even after averaging, the mean image is not blurred. *Right*: The mean image of warped MR images obtained from 47 schizophrenics. The mean image of schizophrenic looks similar to that of controls.

Results

Behavioural data

Patients had a lower full scale IQ, measured by the Wechsler Adult Intelligence Scale—Revised, than controls. They also had a lower expected premorbid IQ measured by a JART, lower scores of Wechsler Memory Scale—Revised and demonstrated poorer performance of working memory measures such as the number of preservative errors in the WCST and digit span (Table 1). No genotype or genotype-diagnosis interaction effects were found in working memory measures

Table 2 Results of image analyses

Anatomical regions	Brodmann area	Cluster size	Corrected P FDR	*T*-value (voxel level)	Talairach coordinates		
					x	*y*	*z*
Main effects							
Diagnosis effects (control > schizophrenia) (Fig. 3)							
Limbic system							
R insula	BA13	4682	0.000	6.41	33	11	−2
L insula	BA13	4017	0.000	8.81	−33	11	4
R parahippocampal gyrus, amygdala-uncus	BA36	4682	0.000	7.32	30	1	−17
R parahippocampal gyrus	BA36	186	0.000	5.04	30	−41	−8
L parahippocampal gyrus, hippocampus-amygdala	BA34/36	637	0.000	5.46	−20	−41	−8
R anterior cingulate cortex	BA32	147	0.000	4.9	9	33	20
L anterior cingulate cortex	BA32	200	0.000	4.63	−11	32	20
L cingulate gyrus	BA32	275	0.001	4.2	−12	−16	39
Prefrontal cortex							
R inferior frontal gyrus	BA47,11	145	0.000	4.99	27	28	−11
R superior frontal gyrus	BA8/9	1889	0.000	6.08	12	43	39
L medial frontal gyrus	BA9	1333	0.000	5.13	−8	47	19
L inferior frontal gyrus	BA45	141	0.000	4.55	−44	23	15
L middle frontal gyrus	BA8	482	0.000	4.44	−30	24	43
L superior frontal gyrus	BA8	482	0.000	4.39	−35	17	51
Premotor area							
R dorsal premotor area	BA6	429	0.000	4.37	41	13	45
Temporal cortex							
R superior temporal gyrus	BA22	806	0.000	5.04	47	−23	−1
R middle temporal gyrus	BA21	806	0.000	4.87	56	−15	−3
L superior temporal gyrus	BA38	4017	0.000	7	−36	1	−17
Central grey matter							
L thalamus		4017	0.000	7.26	−15	−17	2
Diagnosis effects (control < schizophrenia) (Fig. 4)							
L sylvian fissure		621	0.000	6.7	−45	17	−3
R sylvian fissure		774	0.000	6.59	44	17	−8
Lateral ventricle (anterior horn)		279	0.000	5.27	−5	21	4
Lateral ventricle (L inferior horn)		248	0.000	6.18	−41	−30	−10
Lateral ventricle (R inferior horn)		137	0.000	5.02	36	−40	−1
Interhemisphrenic fissure		154	0.000	5.28	3	55	−12
Genotype effects (Val/Val-COMT < Met-COMT carriers) (Fig. 5)							
Limbic system							
L anterior cingulate cortex	BA24/25	334	0.033	4.29	−8	17	−13
Temporal cortex							
R middle temporal gyrus	BA21	285	0.016	5.10	59	−3	−14
Genotype-diagnosis interaction effects (Fig. 6)							
Limbic system							
L anterior cingulate gyrus	BA24/25/32	264	0.044	3.77	−6	25	−6
L parahippocampal gyrus, amygdala-uncus	BA34	219	0.048	3.74	−24	−6	−14
The effects of polymorphism in control group (no significant difference)							
The effects of polymorphism in schizophrenia							
Val/Val-COMT < Val/Met, Met/Met-COMT (Fig. 7)							
Limbic system							
L parahippocampal gyrus, amygdala-uncus	BA28	81	0.010	4.17	−26	2	−22
L anterior cingulate cortex	BA24/25/32	263	0.007	4.38	−7	20	−8
Central grey matter							
L thalamus		91	0.014	3.94	−21	−28	6

and IQ, however, a significant genotype-by-diagnosis interaction effect was found in a visual memory measure (F = 4.605, df = 1, P = 0.03) (Table 1). However, a *post hoc t*-test (Bonferroni test) demonstrated no genotype effect in each diagnostic category (control: P = 0.15, schizophrenia: P = 0.11).

Morphological changes in schizophrenia (diagnosis effects)

In comparison with controls, patients with schizophrenia demonstrated a significant reduction of volumes in multiple brain areas, such as the limbic and paralimbic systems, neocortical areas and the subcortical regions (Table 2 and Fig. 3).

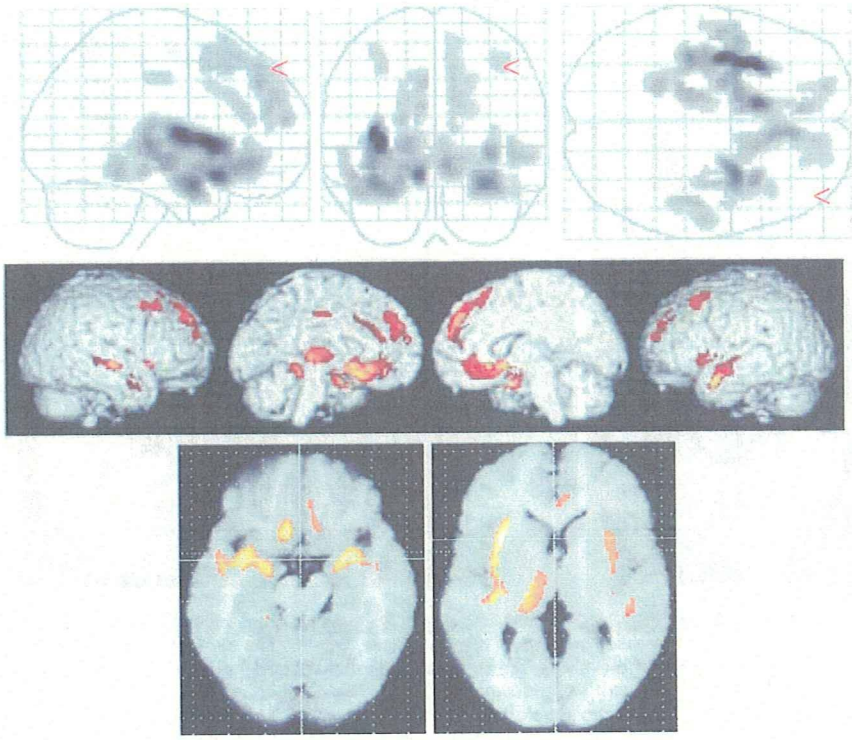

Fig. 3 Decreased volumes in schizophrenics ($n = 47$) as compared to controls ($n = 76$). *Top*: The SPM {*t*} is displayed in a standard format as a maximum-intensity projection (MIP) viewed from the right, the back and the top of the brain. The anatomical space corresponds to the atlas of Talairach and Tournoux. Representation in stereotaxic space of regions with significant reduction of volume in schizophrenia was demonstrated. Schizophrenics demonstrated a significant reduction of volumes in the multiple brain areas, such as the limbic and paralimbic systems, neocortical areas and the subcortical regions. *Middle*: The SPM {*t*} is rendered onto T_1-weighted MR images. *Bottom*: The SPM {*t*} is displayed onto axial T_1-weighted MR images. A significantly decreased volume of the amygdala-uncus, bilateral insular cortices, ACC, temporal cortex and the left thalamus in schizophrenics was noted.

In the limbic and paralimbic systems, patients with schizophrenia showed reduction of volumes in the parahippocampal gyri, amygdala-uncus, insular cortices and the anterior cingulate cortices (ACC). They also demonstrated reduced volumes in the frontal and temporal association areas, dorsal premotor areas and the left thalamus. In comparison with controls, patients with schizophrenia showed significantly increased volume in the CSF space such as lateral ventricle, sylvian and the interhemispheric fissures but not in the grey matter (Table 2 and Fig. 4).

Morphological changes associated with the Val158Met polymorphism (genotype effects)

In comparison with Met-COMT carriers, individuals homozygous for the Val-COMT allele demonstrated a significant reduction of volumes in the left ACC and the right middle temporal gyrus (MTG) (Table 2 and Fig. 5). The hypothesis-driven analysis demonstrated a genotype effect on volumes in the bilateral DLPFC (right BA9, left BA8) at a lenient threshold (uncorrected $P = 0.05$) (data are not shown), however, no voxels could survive after the correction for multiple comparisons (FDR < 0.05) within the ROI. There were no areas that individuals homozygous for the Val-COMT allele demonstrated a significant increment of volume compared to Met-COMT carriers.

Genotype—diagnosis interaction effects

We found significant genotype-diagnosis interaction effects on brain morphology. The stronger effects of Val158Met polymorphism on brain morphology in schizophrenia than those in controls were noted in the left ACC and the left amygdala-uncus (Table 2 and Fig. 6). The hypothesis-driven analysis demonstrated a genotype-diagnosis interaction effect on the volume of the right DLPFC (BA9/46) at a lenient threshold (uncorrected $P = 0.05$) (data not shown), however, no voxels could survive after the correction of multiple comparisons (FDR < 0.05) within the ROI.

Effects of the Val58Met polymorphism on brain morphology

Since genotype–disease interaction effects were found, we estimated the effects of genotypes on brain morphology in the control groups and the schizophrenic groups separately.

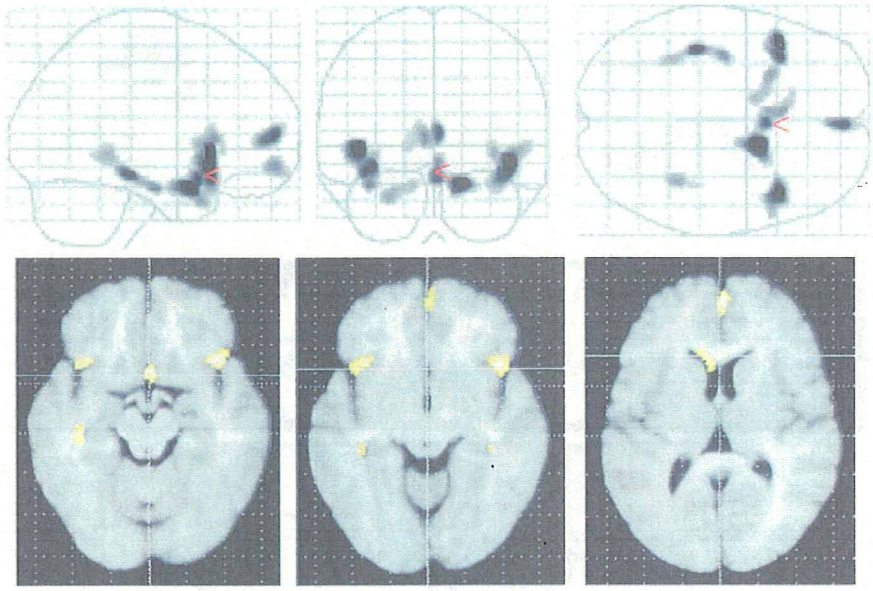

Fig. 4 Increased volumes in schizophrenics as compared to controls. *Top*: The SPM {*t*} is displayed in a standard format as a MIP. Patients with schizophrenia showed a significantly increased volume of the CSF space. *Bottom*: The SPM {*t*} is displayed onto axial T_1-weighted MR images. A significantly increased volume of the CSF space such as the lateral ventricle, sylvian fissures and the interhemisphrenic fissure was noted.

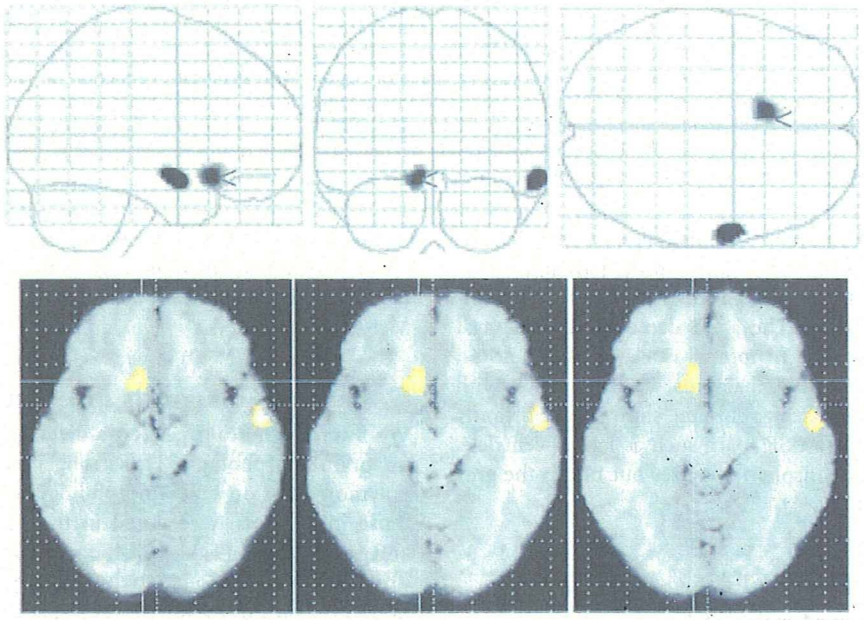

Fig. 5 The result of comparison between individuals homozygous for the Val-COMT allele (n = 57) and Met-COMT carriers (n = 66) (genotype effects). *Top*: Representation in stereotaxic space of regions with significant reduction of volume in individuals homozygous for the Val-COMT allele demonstrated. *Bottom*: The SPM {*t*} is displayed onto axial T_1-weighted MR images. Individuals homozygous for the Val-COMT allele demonstrated a significant reduction of volumes in the left ACC and right MTG as compared to Met-COMT carriers.

In the control group, we found no significant morphological differences between individuals homozygous for the Val-COMT allele and Met-COMT carriers. Even the hypothesis driven analysis with a lenient statistical threshold ($P < 0.05$) could not detect any significant morphological changes in the DLPFC between the two groups. Contrary to the control group, schizophrenics homozygous for the Val-COMT allele showed a significant reduction of volumes in the left amygdala-uncus, bilateral ACC, right MTG and the left thalamus when compared to the patients carrying the Met-COMT

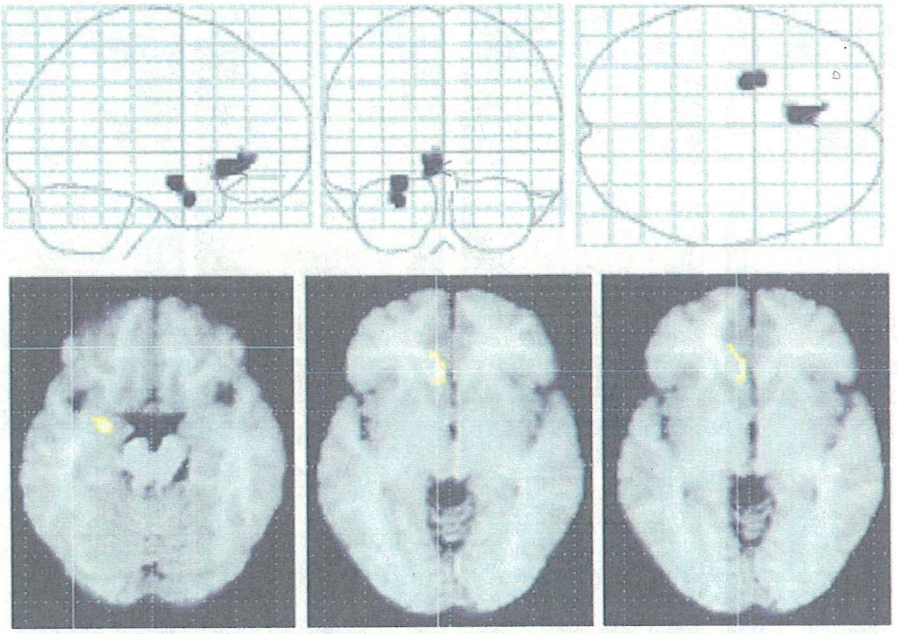

Fig. 6 Results of genotype-diagnosis interaction effects on brain morphology. *Top*: The SPM {*t*} is displayed in a standard format as a MIP. The stronger effects of Val158Met polymorphism on brain morphology in schizophrenia than those in controls were noted in the left ACC, left parahippocampal gyrus and the amygdala-uncus. *Bottom*: The SPM {*t*} is displayed onto axial T_1-weighted MR images.

allele (Table 2, Fig. 7). The hypothesis-driven analysis demonstrated a significantly decreased volume of the bilateral DLPFC in schizophrenics homozygous for the Val-COMT allele when compared to the Met-COMT schizophrenics at a lenient threshold (uncorrected $P = 0.05$) (data not shown). However, no voxels could survive after the correction for multiple comparisons (FDR < 0.05) within the ROI. There are no significantly increased volumes in the schizophrenics homozygous for the Val-COMT allele. All the results were essentially unchanged even if all the left-handed subjects were excluded in all analyses (data not shown).

Discussion

In this study, we found reduction of volumes in the limbic and paralimbic systems, neocortical areas (prefrontal and temporal cortices) and thalamus in patients with schizophrenia when compared to control subjects. The schizophrenia patients demonstrated a significant enlargement of CSF spaces including the lateral and sylvian fissure, which could be interpreted as a result of impaired neurodevelopment and/or global brain atrophy. These findings are concordant with previous studies of MR morphometry of schizophrenia. According to a recent review and meta-analyses of the morphometry of schizophrenia, the consistent abnormalities in schizophrenia are as follows; (i) ventricular enlargement (lateral and third ventricles); (ii) medial temporal lobe involvement; (iii) superior temporal gyrus involvement (iv) parietal lobe involvement; and (v) subcortical brain region involvement including the thalamus (Okubo *et al.*, 2001; Shenton *et al.*, 2001; Davidson and Heinrichs, 2003). The other regions observed in this study, such as the insula, DLPFC and the ACC have also often been demonstrated as abnormal areas in schizophrenia (Shenton *et al.*, 2001; Takahashi *et al.*, 2004; Yamasue *et al.*, 2004). Using the TBM technique, we replicated the morphological abnormalities observed in previous MR studies on schizophrenia, suggesting that TBM was able to detect morphological changes associated with this disease. As well as neuroimaging studies, post-mortem studies have also reported morphological abnormalities in schizophrenia, but not necessarily as common neuropathological features. Regions including the hippocampus, ACC, thalamus and the DLPFC are regularly associated with abnormalities of cell size, cell number and neuronal organization (Bogerts, 1993; Arnold and Trojanowski, 1996; Selemon, 2001; Selemon and Lynn, 2002, 2003). Selemon *et al.* reported that schizophrenics demonstrated abnormalities in overall and laminar neuronal density in the DLPFC (Brodmann area 9) and suggested that the DLPFC should be a particularly vulnerable target in the disease process (Selemon 2001; Selemon and Lynn, 2002, 2003).

Importantly, our results suggest that some of the morphological changes in schizophrenia mentioned above are associated with the Val158Met polymorphism of the COMT gene. In the schizophrenic group, the polymorphism was associated with the volumes in the limbic and paralimbic systems, temporal cortices and the left thalamus, whereas no morphological changes related to the polymorphism were found in

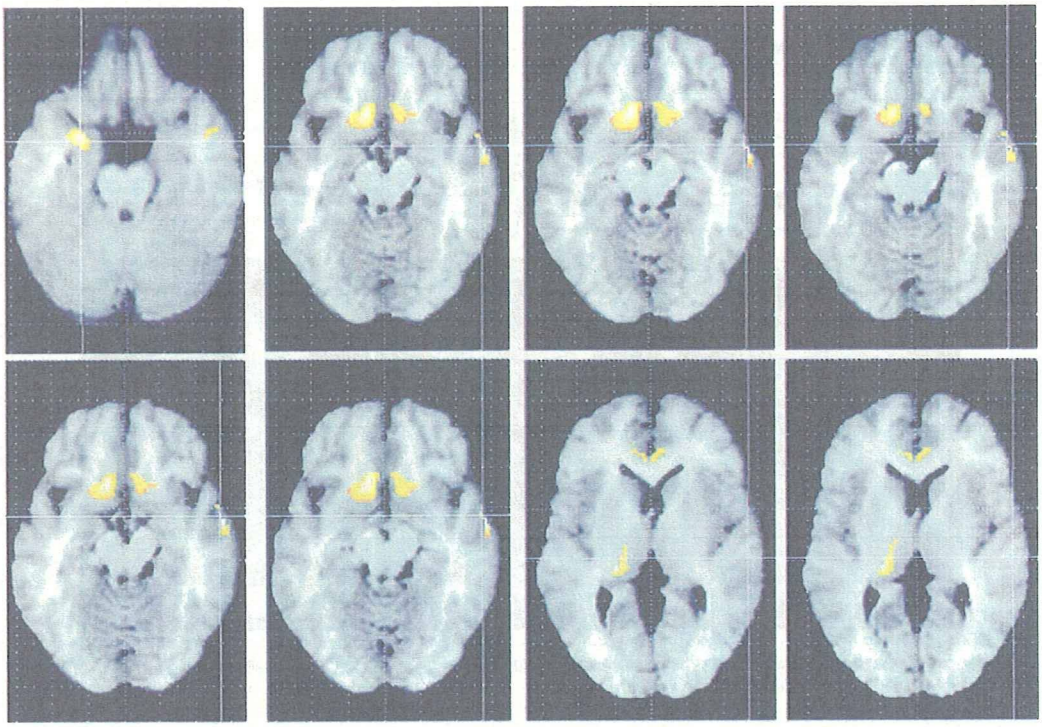

Fig. 7 The effects of the Val158Met polymorphism of the COMT gene on brain morphology in schizophrenics. The SPM {*t*} is displayed onto axial T_1-weighted MR images. The schizophrenics homozygous for the Val-COMT allele (n = 19) showed a significant reduction of volumes in the left parahippocampal gyrus, amygdala-uncus, ACC, left thalamus and the right MTG when compared to patients who carried the Met-COMT allele (n = 28).

normal individuals. As a consequence, significant genotype-diagnosis interaction effects were found in the left ACC and the amygdala-uncus. These results indicate that the Val158-Met polymorphism of the COMT gene is strongly associated with morphological changes in schizophrenia, particularly those in the limbic and paralimbic systems. Longitudinal MRI studies of schizophrenia strongly suggest that progressive changes should occur after onset of the illness (Okubo *et al.*, 2001; Ho *et al.*, 2003). Recent studies have demonstrated that antipsychotic drugs, particularly haloperidol, have considerable effects on brain morphology (Arango *et al.*, 2003; Lieberman, 2005; Dorph *et al.*, 2005). Because of the long duration of illness and medication taken by our subjects, the effects of antipsychotics may be a possible confounding factor for our findings. However, the duration of medication and the dose of antipsychotics taken by the Val/Val-COMT schizophrenics did not differ from those of the Met-COMT schizophrenics. Although the effects of antipsychotics on brain morphology may contribute to the observed morphological changes in patients with schizophrenia in this study, it is unlikely that the effects of antipsychotics contributed to morphological differences between the two schizophrenic groups.

When we were preparing this manuscript, another study demonstrated no genotype and genotype-diagnosis interaction effects of the Val158Met polymorphism on morphology of the frontal lobe in controls and schizophrenia (Ho *et al.*, 2005). Although there are differences between the two studies, such as mean ages of subjects, duration of illness, methods for image analysis and a racial factor (Caucasians versus Japanese), that study also demonstrated no genotype and genotype-diagnosis interaction effects on morphology of the DLPFC. However, we found these effects on DLPFC morphology at a very lenient statistical threshold. Further studies with a larger sample will clarify whether Val158Met polymorphism does affect DLPFC morphology. As well as prefrontal morphology, we found no significant genotype or genotype-diagnosis interaction effects on working memory, however, schizophrenics homozygous for the Val-COMT allele tended to have poorer performances on working memory measures, compared to Met-COMT carriers with schizophrenia. Although there were no significant effects of Val158Met polymorphism on working memory and other neuropsychological measures, a significant effect of the polymorphism was noted in brain morphology. The brain morphology has been considered to be useful as an intermediate phenotype in genetic research in neuropsychiatric disorders (Baare *et al.*, 2001; Durston *et al.*, 2005). Therefore, morphological changes might be more sensitive to the effects of genotype than behavioural measures such as the performance of working memory measures. In a previous study (Ho *et al.*, 2005) a similar phenomenon—no significant effect of Val158Met polymorphism on working memory performance but significant

effects on brain activities during a working memory task—was found. Further studies with a larger sample size are needed to clarify whether morphological changes are a more sensitive marker of genotype effects than behavioural measures.

Unexpectedly, we found effects of the polymorphism on the ACC volume rather than the DLPFC which is crucial for working memory. Since the ACC is associated with a variety of cognitive tasks involving mental efforts, and also plays important roles in working memory (Paus *et al.*, 2001; Kondo *et al.*, 2004), it is feasible that the Val158Met polymorphism may be associated with the ACC morphology. In fact, a previous study demonstrated that the Val-COMT allele was associated with abnormal ACC function as well as abnormal prefrontal cortical function, relative to the Met-COMT allele, as measured by cognitive tests and fMRI activation in normal subjects (Egan *et al.*, 2001).

One would argue that the effects of one polymorphism of the gene could not explain the morphological changes in schizophrenia. As well as the effects of the Val158Met polymorphism, we agree that other polymorphisms of schizophrenia susceptibility genes and genotype–genotype interaction may relate to individual brain morphology. Such interactions might contribute to the different effects of the Val158Met polymorphism on brain morphology observed in this study. Further studies of each effect and interaction of several schizophrenia susceptibility genes on brain morphology, brain functions and performances of neuropsychological tests should be conducted to clarify how polymorphisms of these genes affect intermediate phenotypes of schizophrenia.

In conclusion, we found an association between the Val158Met polymorphism and morphological abnormalities in schizophrenia. Although the underlying mechanisms of our observation remain to be clarified, our data indicate that brain morphology as an intermediate phenotype should be useful for investigating how genotypes affect endophenotypes of schizophrenia.

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Abstract—There is no specific treatment to improve the functional recovery in the chronic stage of ischemic stroke. To provide the new therapeutic options, we examined the effect of overexpression of hepatocyte growth factor (HGF) in the chronic stage of cerebral infarction by transferring the HGF gene into the brain using hemagglutinating virus of Japan envelope vector. Sixty rats were exposed to permanent middle cerebral artery occlusion (day 1). Based on the sensorimotor deficits at day 7, the rats were divided equally into control vector or HGF-treated rats. At day 56, rats transfected with the HGF gene showed a significant recovery of learning and memory in Morris water maze tests (control vector 50±4 s; HGF 33±5 s; $P<0.05$) and passive avoidance task (control vector 132.4±37.5 s; HGF 214.8±26.5 s; $P<0.05$). Although the total volume of cerebral infarction was not related to the outcome, immunohistochemical analysis for Cdc42 and synaptophysin in the peri-infarct region revealed that HGF enhanced the neurite extension and increased synapses. Immunohistochemistry for glial fibriary acidic protein revealed that the formation of glial scar was also prevented by HGF gene treatment. Additionally, the number of the arteries was increased in the HGF group at day 56. These data demonstrated that HGF has a pivotal role for the functional recovery after cerebral infarction through neuritogenesis, improved microcirculation, and the prevention of gliosis. Our results also provide evidence for the feasibility of gene therapy in the chronic stage of cerebral infarction. (*Hypertension*. 2006;47:742-751.)

Key Words: cerebral ischemia ■ genes ■ microcirculation ■ rats

Middle cerebral artery occlusion (MCAo) is one of the most common causes of focal stroke in humans[1] and causes severe sensorimotor deficits and cognitive dysfunction. The ischemic changes closely resemble those produced in a MCAo model in rats,[1] which causes infarction mainly in the dorsolateral and lateral portions of the neocortex and the entire caudoputamen.[2] Several growth factors are upregulated immediately after MCAo, such as fibroblast growth factor (FGF),[3] brain-derived neurotrophic factor,[4] glial cell line-derived neurotrophic factor,[5] vascular endothelial growth factor (VEGF),[6] and hepatocyte growth factor (HGF),[7] and thought to protect neurons or promote angiogenesis after MCAo. In fact, the extension of infarction is prevented by administration of growth factors or gene transfer of growth factors before or immediately after MCAo.[8–11] However, the therapeutic time window of such treatment is too short for clinical use,[12] because they focused on preventing the extension of neuronal death in the penumbra in the acute stage.

Recently, HGF and c-Met/HGF have been reported to be upregulated mainly in the peri-infarct region as long as 28 days after permanent MCAo[13] and up to 14 days in FGF[3] or VEGF.[14] HGF is a well-known potent pleiotropic cytokine that exhibits mitogenic, motogenic, and morphogenic activity in a variety of cells.[15–17] Both HGF and the c-Met/HGF receptor of membranes spanning tyrosine kinase are expressed in various regions of the brain.[17] HGF is also involved in the development and maintenance of cortical neurons during differentiation, motogenesis, neuritogenesis, and neuronal survival during the development of the rat cerebral cortex.[18] Interestingly, HGF promotes proliferation and neuronal differentiation of neural stem cells from mouse embryos.[19] In vivo, it has also been demonstrated that HGF promotes angiogenesis in cerebral ischemia in rodents[20–23] without disrupting the blood–brain barrier.[10]

From these viewpoints, we speculated that HGF might play a pivotal role in the functional recovery in the chronic stage of ischemic insult, and its overproduction could improve the cognitive dysfunction. To clarify this speculation, we transferred the human HGF gene into the brain 7 days after MCAo, using the hemagglutinating virus of Japan (HVJ)-

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envelope vector,[10] and examined behavioral tests, MRI, and histological changes. Here, we demonstrated that gene therapy delayed for as long as 7 days improved outcome from ischemic stroke, and HGF is an important growth factor for the recovery of cognitive function in the chronic stage of MCAo through reconstitution of the neuronal network.

Methods

Preparation of HVJ-Envelope Vector

HVJ-envelope vector was prepared as described previously.[24,25] Briefly, virus suspension (15 000 hemagglutinating units) was inactivated by UV irradiation (99 mJ/cm^2) and mixed with plasmid DNA (400 μg) and 0.3% Triton-X. After centrifugation, it was washed with 1 mL of balanced salt solution (10 mmol/L Tris-Cl (pH 7.5), 137 mmol/L NaCl, and 5.4 mmol/L KCl) to remove the detergent and unincorporated DNA. After centrifugation, the envelope vector was suspended in 100 mL of PBS. The vector was stored at 4°C until use.

Construction of Plasmids

To produce an HGF expression vector, human HGF cDNA (2.2 kb) was inserted into a simple eukaryotic expression plasmid that uses the cytomegalovirus promoter/enhancer.[26] This promoter/enhancer has been used to express reporter genes in a variety of cell types and can be considered constitutive. The control vector had the same structure as the expression vector plasmid, including the promoter but not containing HGF cDNA. Plasmids were purified with a QIAGEN plasmid isolation kit (Qiagen).

Surgical Procedure

Male Wistar rats (270 to 300 g; Charles River Japan, Atsugi, Japan) were used in this study. To generate a permanent MCAo model, the right middle cerebral artery (MCA) was occluded by placement of poly-L-lysine-coated 4-0 nylon around the origin of the MCA, as described previously.[2] In vivo gene transfer was performed by intracisternal injection as described previously.[25] Briefly, rats were anesthetized with ketamine (Sankyo) and xylazine (Bayer Ltd). HVJ-envelope vector (100 μL) containing the human HGF gene was infused at 50 μL/min after removing 100 μL of cerebrospinal fluid (CSF). The protocol was approved by the Committee on the Ethics of Animal Experiments in the Osaka University. To examine transfection of the HGF gene in the CSF, CSF (100 μL) was collected 4, 7, 14, and 21 days after gene transfer. The concentration of HGF was determined by enzyme immunoassay using anti-human HGF antibody (Institute of Immunology, Tokyo, Japan) as described previously.[10]

Protocol for Treatment and Behavioral Tests

Ten rats were only anesthetized (sham operation), and 60 rats were subjected to MCAo (day 1). Based on the neuromuscular function and body weight evaluated on day 7, the rats were divided equally into control vector-treated (n=23) and HGF-treated (n=23) groups. Rats showing no palsy on day 7 or that died before day 7 were excluded from the present study (n=14). On day 55, neuromuscular function and locomotor activity were evaluated in the surviving rats (n=20 for control vector-treated and n=22 for HGF-treated rats). Then, cognitive function was examined by Morris water maze (MWM) and passive avoidance task from day 56 to 90. On day 96, MRI was performed to evaluate the volume of infarction.

Sensorimotor Deficit and Locomotor Activity

Although there are various batteries for testing sensorimotor deficit, we used a simple protocol[27] to evaluate sensorimotor deficit, which used the following categories (maximum score is 4). For forelimb flexion, rats were held by the tail on a flat surface. Paralysis of the forelimbs was evaluated by the degree of left forelimb flexion. For torso twisting, rats were held by the tail on a flat surface. The degree of body rotation was checked. For lateral push, rats were pushed either left or right. Rats with right MCA occlusion showed weak or no resistance against a left push. For hind limb placement, one hind limb was removed from the surface. Spontaneous locomotor activity was also measured via the open field test for 30 minutes using an automated activity box (Muromachi Kikai).

MWM Task

A cylindrical tank 1.5 m in diameter was filled with water (25°C), and a transparent platform 15 cm in diameter was placed at a fixed position in the center of 1 of the 4 quadrants (O'Hara & Co, Ltd). In the hidden platform test, the platform was set below the water level, and it was not seen by the rats. The platform was fixed at 1 quadrant, and the starting point was changed in each trial. A previous study showed a difference in the latency of reaching the platform until day 6 of the session between rats exposed to MCAo 12 to 14 weeks before and control rats, if the tests were performed twice a day.[28] Based on the results, we carried out the tests twice a day for 6 days. If the rat could not reach the platform, the latency was set at 60 s. In the visible platform test, a flag was placed on the platform, which could be seen by the rats. The tests were carried out twice a day for 6 days. In this trial, the platform and the starting point were changed in each trial. Throughout the tests, the path of swimming was captured by a charge-coupled device video camera and analyzed by National Institute of Health image.

Passive Avoidance Task

A step-through type of passive avoidance task was used in the present study. The apparatus (Medical Agent) consisted of an illuminated chamber and a dark one. To habituate the rats, they were placed in the illuminated chamber, and the door was opened so that they could enter the dark one. Rats have a habit of entering the dark chamber, because they prefer darkness. In an acquisition trial, the rats were placed in the illuminated chamber and exposed to a 0.6-mA foot shock when they entered the dark chamber. Each trial was continued until the rat learned not to enter the dark chamber for 300 seconds. In retention trials, they were placed in the illuminated room 3 days after the acquisition trial. We evaluated the latency (maximum: 300 s) of their staying in the illuminated room.

Immunohistochemical Study

For histopathologic analysis, other rats (control vector-treated [n=4] or HGF-treated [n=4] rats in each experiment) were treated the same as described above and euthanized on day 11, 14, or 56, followed by transcardial perfusion fixation with normal saline followed by 4% paraformaldehyde. The brain was removed, postfixed, cryoprotected, and cut on a cryostat at 12 μm. After blocking, sections were incubated in 3% normal goat serum and anti-MAP2 (1:1000; mouse monoclonal; Sigma-Aldrich, St Louis, MO), GFAP (1:1000; mouse monoclonal; Sigma-Aldrich), and Cde42 (1:100; mouse monoclonal; Santa Cruz Biotechnology, Santa Cruz, CA) followed by anti-mouse goat fluorescent antibody (1:1000 for MAP2 and GFAP, 1:250 for Cde42, Alexa Flour 546, Molecular Probes). For immunostaining of human HGF or synaptophysin, sections were treated with 2% H_2O_2 to block endogenous peroxidase and then incubated with an antibody against human HGF-β (H714; 1:250; rabbit polyclonal; Immuno-Biological Laboratories, Gunma, Japan) or synaptophysin (1:500; mouse monoclonal; Chemicon, Temecula, CA) at 4°C O/N. They were incubated with streptavidin-horseradish peroxidase (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA), and the biotin-streptavidin-peroxidase complex was detected with diaminobenzidine (human HGF) or tetramethylbenzidine (synaptophysin) peroxidase substrate solution (Vector Laboratories). Negative control sections from each animal received identical preparations for immunohistochemical staining, except that primary antibodies were omitted.

Quantitative Histological Analysis

To quantify the immunoreactivity for GFAP and synaptophysin, the acquired image was imported into Adobe Photoshop (version 7.0, Adobe System). The color image was converted into a grayscale image. This was imported into Mac SCOPE (version 2.5, Mitani Corporation). The region of interest was set at the peri-infarct region

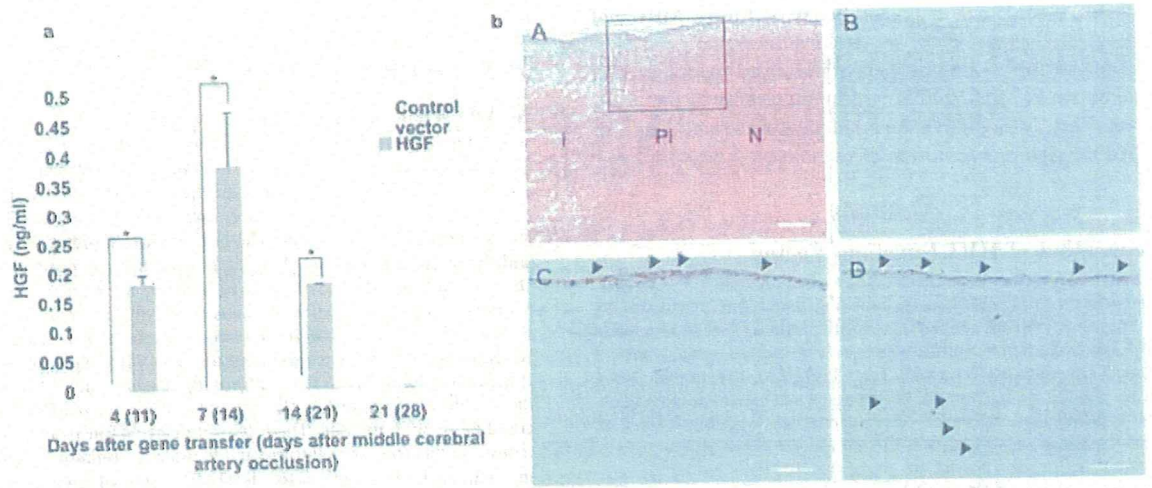


Figure 1. (a) Concentrations of human HGF in cerebrospinal fluid at 4, 7, 14, and 21 days after gene transfer (11, 14, 21, and 28 days after middle cerebral artery occlusion). Control vector indicates rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). *$P<0.01$ vs Control. (b, part A) HE staining at 4 days after gene transfer (11 days after middle cerebral artery occlusion). I, infarct region; PI, peri-infarct region; N, normal region. Bar=100 μm. (B through D) Representative images of immunohistochemical staining for human HGF. (B) Peri-infarct region in rats transfected with control vector (rectangle area in A). Bar=50 μm. (C) Contralateral intact region in rats transfected with HGF vector. Bar=50 μm. (D) Peri-infarct region in rats transfected with HGF vector. Arrowhead showed immunopositive cells for human HGF. Bar=50 μm.

in the cerebral neocortex. The peri-infarct region is defined as the area surrounding the lesion, which morphologically differs from the surrounding normal tissue (Figure 1b, part A).[29,30] The number of pixels for which the signal was >25 was counted. Immunoreactivity was calculated by the equation: % Area=(Number of high signal pixels)/(Total number of pixels). To quantify the cerebral edema, we calculated the percentage of measured infarct area in the corrected infarct area at 0.7 mm from bregma. The corrected infarct area was calculated as [LT-(RT-RI)], where LT is the area of the left hemisphere, RT is the area of the right hemisphere, and RI is the infarct area.[10] The infarct region is edematous when the percentage is >100%. The infarct brain is atrophic if the percentage is <100%.

Alkaline Phosphatase Staining

For alkaline phosphatase (ALP) staining, sections were washed in Tris-HCl and incubated for 30 minutes in substrate solution (a mixture of naphtol AS-BI phosphate [σ-Aldrich] and fast red violet LB salt [σ-Aldrich]). Five consecutive sections in each rat were observed, and acquired images were imported into Adobe Photoshop. The color image was converted into a grayscale image. Then, the ROI was set as the region in the peri-infarct region. The area or length of vessels was analyzed with an Angiogenesis Image Analyzer (version 1.0, Kurabo).

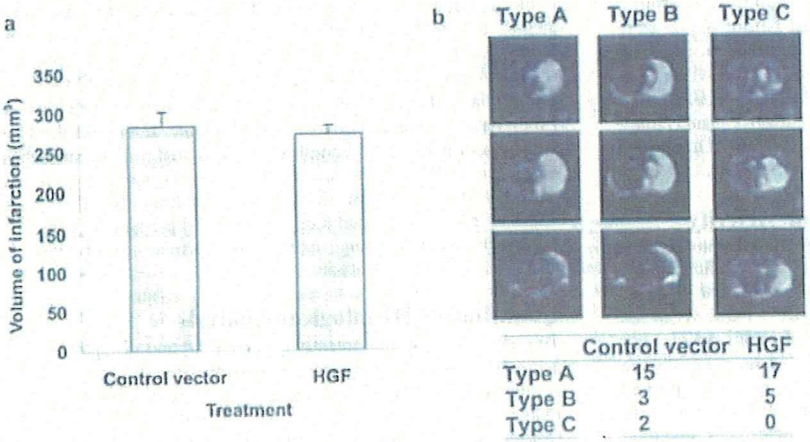
Statistical Analysis

All of the values are expressed as mean±SEM. ANOVA was used to determine the significance of differences in multiple comparisons. $P<0.05$ was considered significant.

Results

Transfer of HGF Gene Improves Learning and Memory After Cerebral Infarction

To test for successful gene transfer via the subarachnoid space, the concentration of human HGF in CSF was measured by ELISA at 4, 7, 14, and 21 days after gene transfer. As expected, human HGF could be detected in the CSF of rats transfected with human HGF vector 4 at 7 days after gene transfer, whereas human HGF protein could not be detected in control rats (Figure 1a). Human HGF protein was detected in the pia mater in the



	Control vector	HGF
Type A	15	17
Type B	3	5
Type C	2	0

Figure 2. Magnetic resonance images of brain. (a) Volume of infarction in all rats calculated in T2-weighted images. Control vector indicates rats transfected with control vector (n=20); HGF, rats transfected with HGF vector (n=22). (b) Typical T2-weighted image of coronal section of rat brain. The images were divided into 3 groups: types A, B, and C (described in text). Most rats showed type A, and fewer showed type B or type C.

normal region (Figure 1b, part C), as well as in the pia mater and parenchyma in the infarct and peri-infarct region 4 days after gene transfer using immunohistochemistry (Figure 1b, part D). Although HE staining at 4 days after gene transfer showed that the infarct brain is atrophic in this timing, as reported previously,[31] there was no significant difference between rats transfected with the human HGF gene and control vector (control vector 87.1±8.1%, HGF 81.0±4.3%; P value not significant).

To confirm the severity of cerebral infarction, all of the rats were examined by T2-weighted MRI on day 96. Although the total volume of infarction calculated in T2-weighted images was not different between rats transfected with the human HGF gene and control vector (Figure 2a), the pattern of cerebral infarction was divided into 3 groups: (1) type A, high-intensity area seen in the dorsolateral and lateral portions of neocortex and the entire caudoputamen; (2) type B, high-intensity area seen in the dorsolateral and lateral portions of neocortex and in part of the caudoputamen; and (3) type C, high-intensity area seen in part of the lateral neocortex and caudoputamen (Figure 2b). In type C, most part of lateral neocortex was intact.

In the hidden platform test of MWM, which examined spatial learning and memory, the latency in rats transfected with control vector was markedly longer as compared with sham-operated rats, and the latency in rats transfected with HGF vector was significantly shorter than that of rats transfected with control vector (Figure 3a). There were no differences both in swimming speed and visible platform test, which excluded the possible influences of visual loss, sensorimotor deficit, and motivation on the results,[32] between rats transfected with control and HGF vector (data not shown). Thus, spatial learning and memory partly, but significantly, recovered in rats transfected with HGF vector. In the passive avoidance task, which was used to measure associated learning and memory,[32] the retention of memory was longer in rats transfected with the HGF vector (Figure 3b), which demonstrated a trend toward significance (P=0.053). Sensorimotor deficit and locomotor activity were also tested, because they have some influence on tests of cognitive function.[32] Sensorimotor deficit had spontaneously recovered to some extent by day 55 in both groups, and there was no difference between the

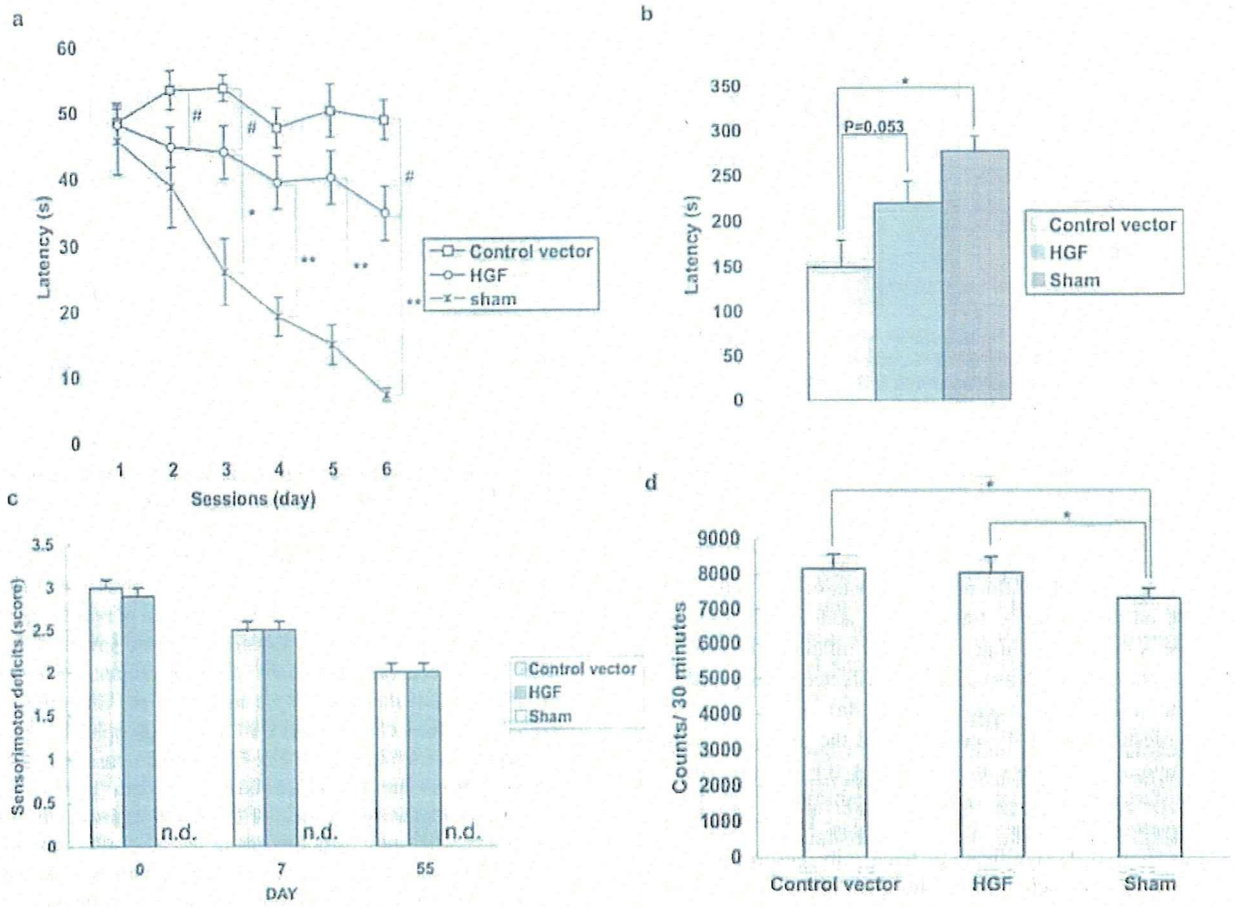


Figure 3. Learning and memory in the chronic stage of cerebral infarction. (a) Hidden platform test in MWM test in all rats. Although rats subjected to middle cerebral artery occlusion hardly reached the hidden platform as compared with sham-operated rats, rats transfected HGF vector could reach faster than that of control vector. (b) Retention trial in passive avoidance task in all rats. The latency of rats staying in the illuminated chamber was calculated. (c) Sensorimotor deficit and (d) spontaneous locomotor activity in all rats. There is no sensorimotor deficit in sham-operation rats in "c" (shown as "n.d."). Control vector indicates rats transfected with control vector (n=20); HGF, rats transfected with HGF vector (n=22); Sham, sham-operated rats (n=10); $^{*}P<0.05$, $^{**}P<0.01$ vs Sham, $^{\#}P<0.05$ vs Control.

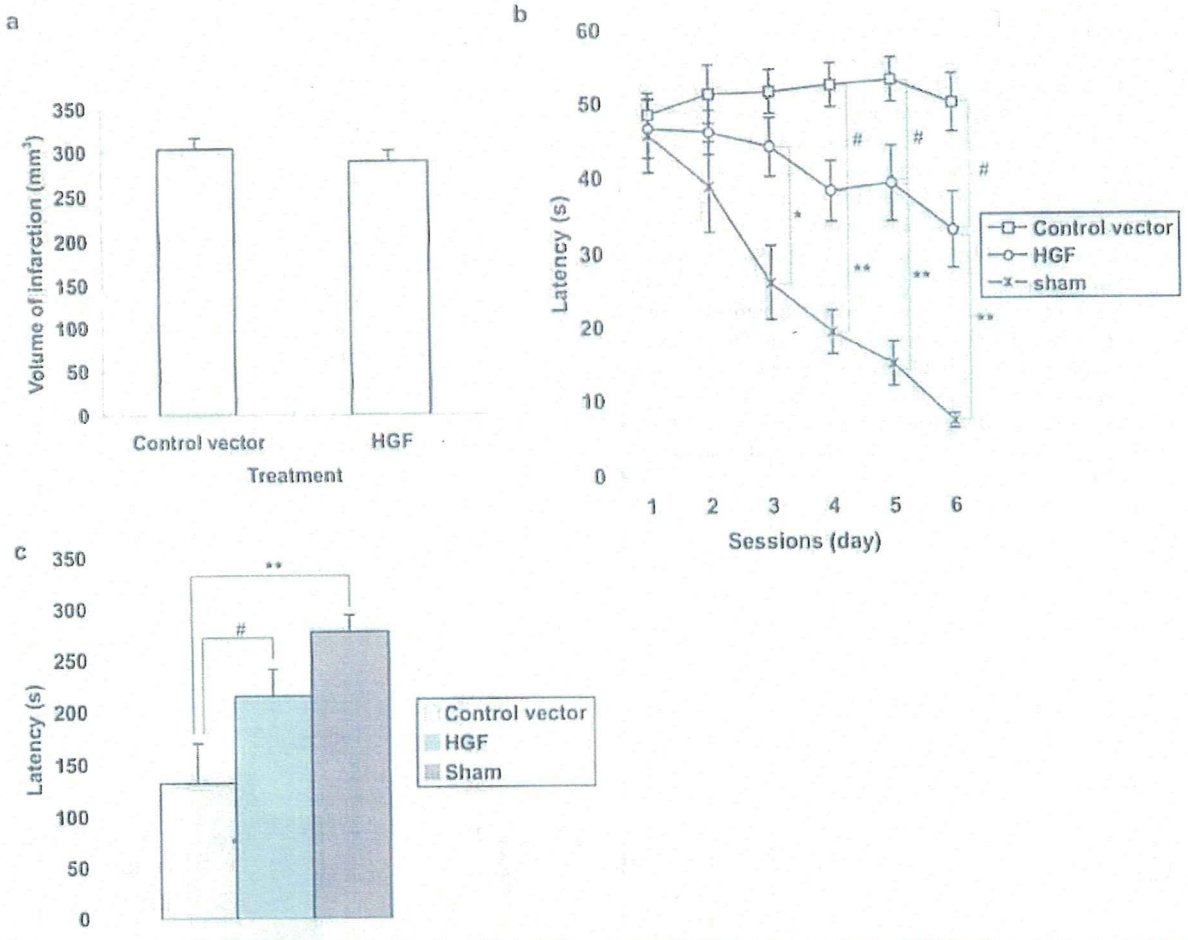


Figure 4. (a) Volume of infarction in type A rats. (b) Hidden platform test in MWM test in type A rats. (c) Retention trial in passive avoidance task in type A rats. Control vector indicates rats transfected with control vector (n=15); HGF, rats transfected with HGF vector (n=17); Sham, sham-operated rats (n=10). *P<0.05, **P<0.01 vs Sham, #P<0.05 vs Control.

2 groups (Figure 3c). Locomotor activity of rats subjected to MCAo was increased as compared with sham-operated rats, as described before,[33] but there was no difference in rats transfected with control and HGF vector (Figure 3d).

To exclude the influence of the pattern of cerebral infarction on the cognitive function, we additionally focused on type A rats. The volume of cerebral infarction in type A rats was not different between rats transfected with human HGF gene and control vector (Figure 4a). Even type A rats transfected with HGF vector showed the improvement in the learning and memory in MWM test (Figure 4b). Also, rats transfected HGF vector showed the significantly longer retention of memory in the passive avoidance task (Figure 4c). Type A rats showed no significant difference in sensorimotor deficit and locomotor activity (data not shown).

These data supported the results that in the MWM and passive avoidance task were not influenced by the sensorimotor and locomotor activity and the volume and pattern of cerebral infarction. Overall, these data suggest that rats transfected with HGF vector maintain their memory longer as compared with those transfected with control vector.

HGF Enhances Neuritogenesis and Synaptogenesis

To examine whether HGF induced neuritogenesis and/or synaptogenesis, we focused on Cdc42, which belongs to the Rho family of GTPases and has positive effects on neuronal process extension,[34] and synaptophysin, which is used as presynaptic markers and synaptogenesis.[35,36] According to previous reports that the neuronal process extension occurred until 14 days after focal cerebral ischemia[36] and synaptogenesis in the chronic stage of the insult,[36] we measured the immunopositive cells against Cdc42 at day 14 and synaptophysin at day 56. Although the number of Cdc42-positive neurons was the same in the contralateral neocortex in both groups, the peri-infarct region in the neocortex of rats transfected with the HGF vector showed a significant increase in the number of Cdc42-immunoreactive cells (Figure 5a and 5b). Also, the immunoreactivity of synaptophysin was significantly increased at day 56 in rats transfected with the HGF gene, especially in the peri-infarct region (Figure 5c and 5d).

HGF Prevents Glial Scar Formation

Then, we investigated whether HGF had influences on astrocytes, because the neuron–glia interaction is also impor-

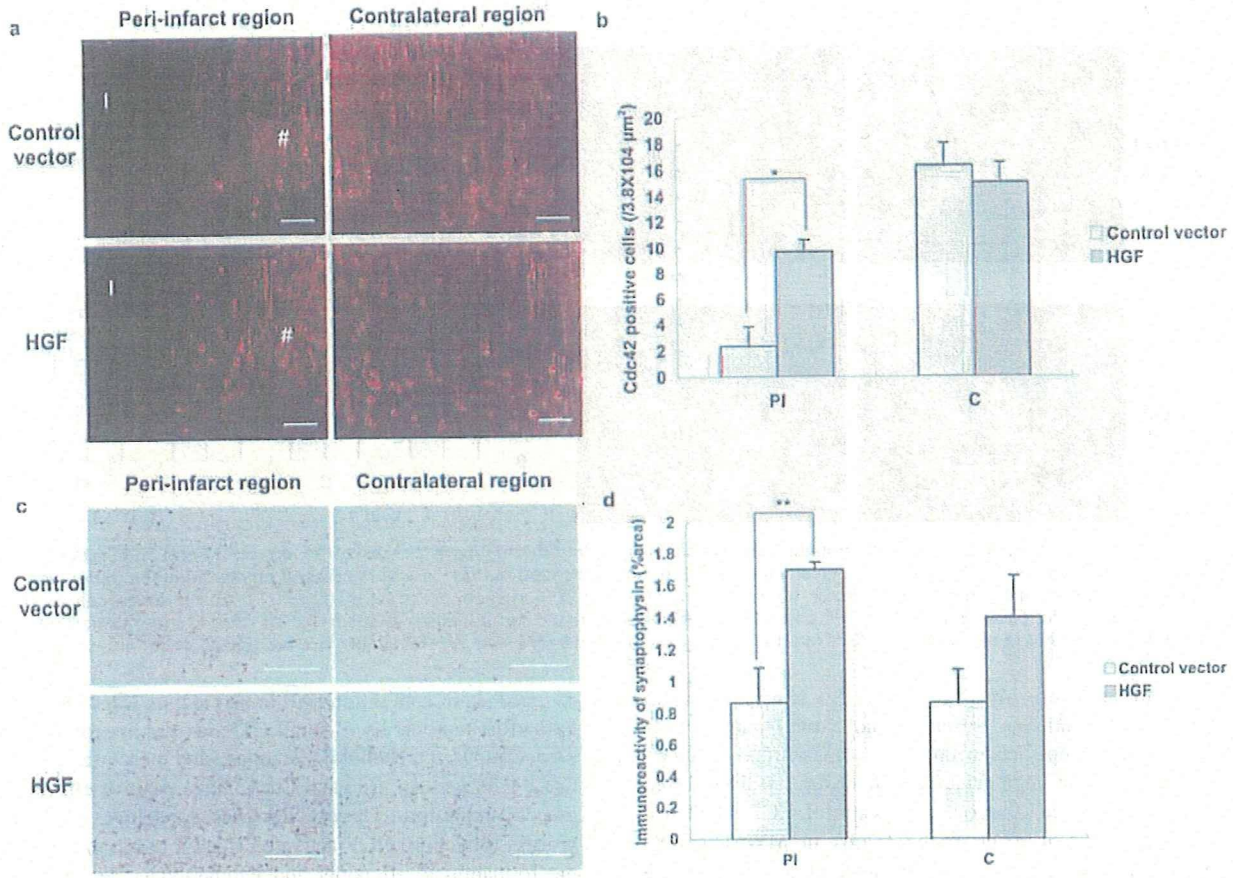


Figure 5. (a) Representative images of immunohistochemical staining for Cdc42 on day 14 in rats transfected with control and HGF vector. The number of cells immunoreactive for Cdc42 was significantly increased in the pyramidal neurons in the peri-infarct region (#) of rats transfected with HGF vector. I, infarct region. (b) Quantitative analysis for Cdc42-immunoreactive cells in peri-infarct region (#). (c) Typical images of immunohistochemical staining for synaptophysin on day 56 (●). (d) Quantitative analysis for the immunoreactivity of synaptophysin. In the peri-infarct region, the immunoreactivity was significantly increased in rats treated with HGF gene. Control vector indicates rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). $^{*}P<0.05$, $^{**}P<0.01$ vs Control. Bar=100 μm. PI, peri-infarct region in neocortex; C, contralateral region in neocortex.

tant for neuroprotection or neuritogenesis.[37] The immunoreactivity of GFAP was increased on days 14 and 56 in the peri-infarct region in both groups, and the immunoreactivity on day 14 was significantly higher in rats transfected with HGF vector (Figure 6). In contrast, the fewer immunopositive cells against GFAP could be detected in rats transfected with the HGF vector on day 56 as compared with the control vector (Figure 6).

Because some viral vectors, such as adenoviral vector, cause diffuse encephaloventriculitis and substantial leukoencephalopathy,[38] we also performed hematoxylin/eosin staining to examine the inflammation. As expected, there was no inflammatory lymphocyte infiltration in HGF and control vector–transfected rats compared with sham-operated rats (data not shown).

HGF Increases Microvessels in the Peri-Infarct Region

Finally, the arteries in the peri-infarct and contralateral region were also examined using ALP staining on days 14 and 56. In the peri-infarct region, on day 56, arteries were significantly increased in rats transfected with HGF vector as compared with control vector (Figure 7a). Consistently, quantitative analysis showed an increase in the area and length of arteries on day 56 in the peri-infarct region in rats transfected with HGF vector (Figure 7b and 7c). Of importance, in the contralateral region, there was no difference between the groups on days 14 and 56 (Figure 7b and 7c).

Discussion

Disruption of blood flow to the brain initiates a cascade of events that produces neuronal death and leads to neurological dysfunction. From this viewpoint, we and others have reported that pretreatment with neurotrophic factors, such as FGF and HGF, has beneficial effects to prevent brain injury. However, considering their clinical application, pretreatment with neurotrophic factors might not be feasible. Unfortunately, few reports have revealed beneficial effects of treatment after infarction. To develop new therapeutic strategies to treat brain infarction, in this study, we examined the effects of overexpression of HGF after infarction, because HGF has unique actions in the central nervous system, as (1) a survival

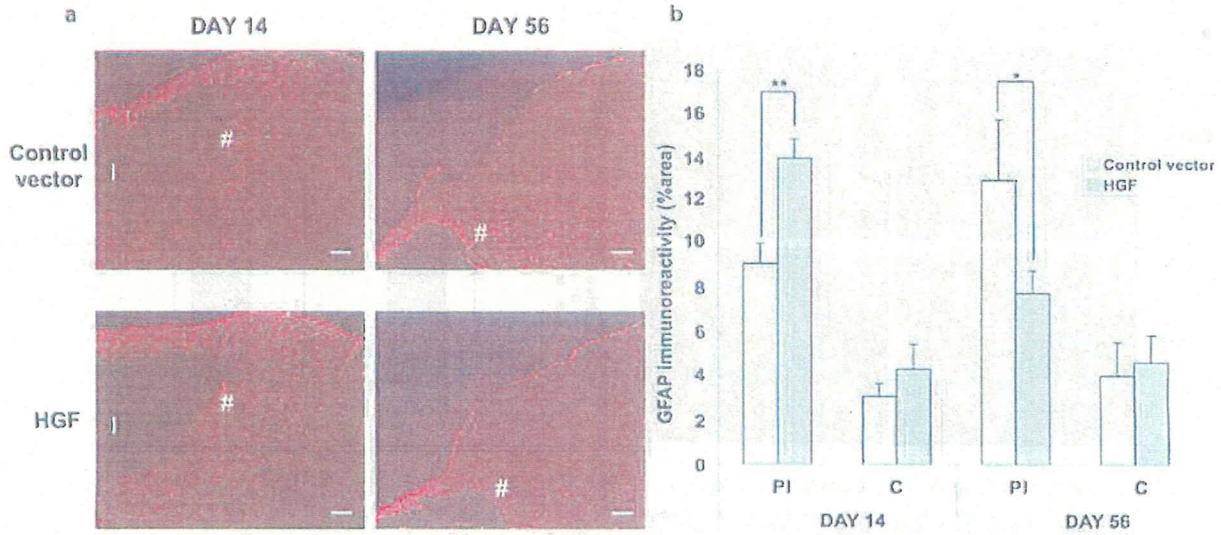


Figure 6. (a) Representative images of immunohistochemical staining for GFAP in ipsilateral neocortex on day 14 and 56 in rats transfected with control and HGF vector. Positive staining for GFAP was increased on day 14 and decreased on day 56 in the peri-infarct region (#) in rats transfected with HGF vector. I indicates infarct region (n=4 in each group; bar=100 μm.) (b) Quantitative analysis of immunoreactivity for GFAP in neocortex. PI, peri-infarct region in neocortex; C, contralateral region in neocortex. Control vector, rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). *P<0.05, **P<0.01 vs Control.

factor for embryonic motor neurons; (2) a stimulatory factor for the differentiation, survival, and axonal outgrowth of sensory and sympathetic neurons; (3) a neurotrophic factor;[39] and (4) a potent angiogenic growth factor.[10,21] The present study demonstrated that overexpression of HGF resulted in significant improvement of the results in MWM and the passive avoidance task on day 56, without any difference in infarct size and pattern. This study demonstrated that treatment with HGF postinfarction improved learning and memory.

Interestingly, the overexpression of HGF did not act on the disability of sensorimotor function and locomotor activity. The discrepancy of the recovery between the sensorimotor and cognitive functions has also been reported recently.[40] The authors reported that the functional recovery was observed not in the cognitive function but in the sensorimotor deficits when MHP36 stem cells were grafted into the cerebral parenchyma, whereas only spatial learning was improved in rats with intraventricular grafts.[40] Although the reason why the discrepancy was caused was unclear in the present study, we speculate that the functional recovery might be dependent on the kind of growth factor or the route of administration because of the different mechanisms in recovery from sensorimotor and cognitive deficits. Because the improvement of the sensorimotor deficits is also important, additional study is necessary to achieve the improvement of sensorimotor deficits.

The region where the significant histological difference was observed was the peri-infarct region, which was the border region between the frontal and parietal cortex. Because the neocortex was also an important site for learning and memory,[41] we speculate that the functional recovery enhanced by HGF is dependent on that region in the present study. In fact, both the immunohistochemical analysis for Cdc42, synaptophysin, and GFAP and the ALP staining revealed significant differences in that region. Cdc42 belongs to the Ras superfamily of small GTPases and is expressed in hippocampus, cerebellum, thalamus, and neocortex in the rats.[34,42] In general, Rac and Cdc42 have positive effects on process extension, whereas Rho has a positive effect on process retraction. HGF activated Cdc42, concomitant with the formation of filopodia and lammelipodia, in epithelial cells,[43] although it was not still demonstrated in neurons. Considering that the immunoreactivity for Cdc42 in pyramidal neurons, which possess a high density of cholinergic terminals,[41] was enhanced at day 14, the reconstitution of the neural network through neurite extension, so-called "neuritogenesis," might be in progress at the early stage of HGF gene treatment. Also, the immunoreactivity of the presynaptic marker synaptophysin was increased at day 56 in rats treated with the HGF gene, which implies that the neuritogenesis resulted in the formation of new synapses.[35,36] These results suggested that HGF enhanced neuritogenesis and synaptogenesis, which might contribute to the recovery of cognitive dysfunction.

The association of neurogenesis is also the center of interest, because HGF is involved in the development and maintenance of cortical neurons during differentiation and motogenesis in the neocortex.[18] In general, adult neurogenesis in the neocortex is still controversial.[44,45] It is also unclear whether adult neurogenesis occurs in the neocortex in rats after focal cerebral ischemia, because Jiang et al[46] showed the existence of neurogenesis, but Zhang et al[47] failed to detect neuronal nuclei and 5-bromodeoxyuridine double-labeling cells in the neocortex. In the present study, the fact that the volume of infarction was not decreased by transfection of the HGF gene and the density of matured neurons assessed by immunohistochemistry for MAP2 was not different (data not shown) implied that neurogenesis was not related to the functional recovery.

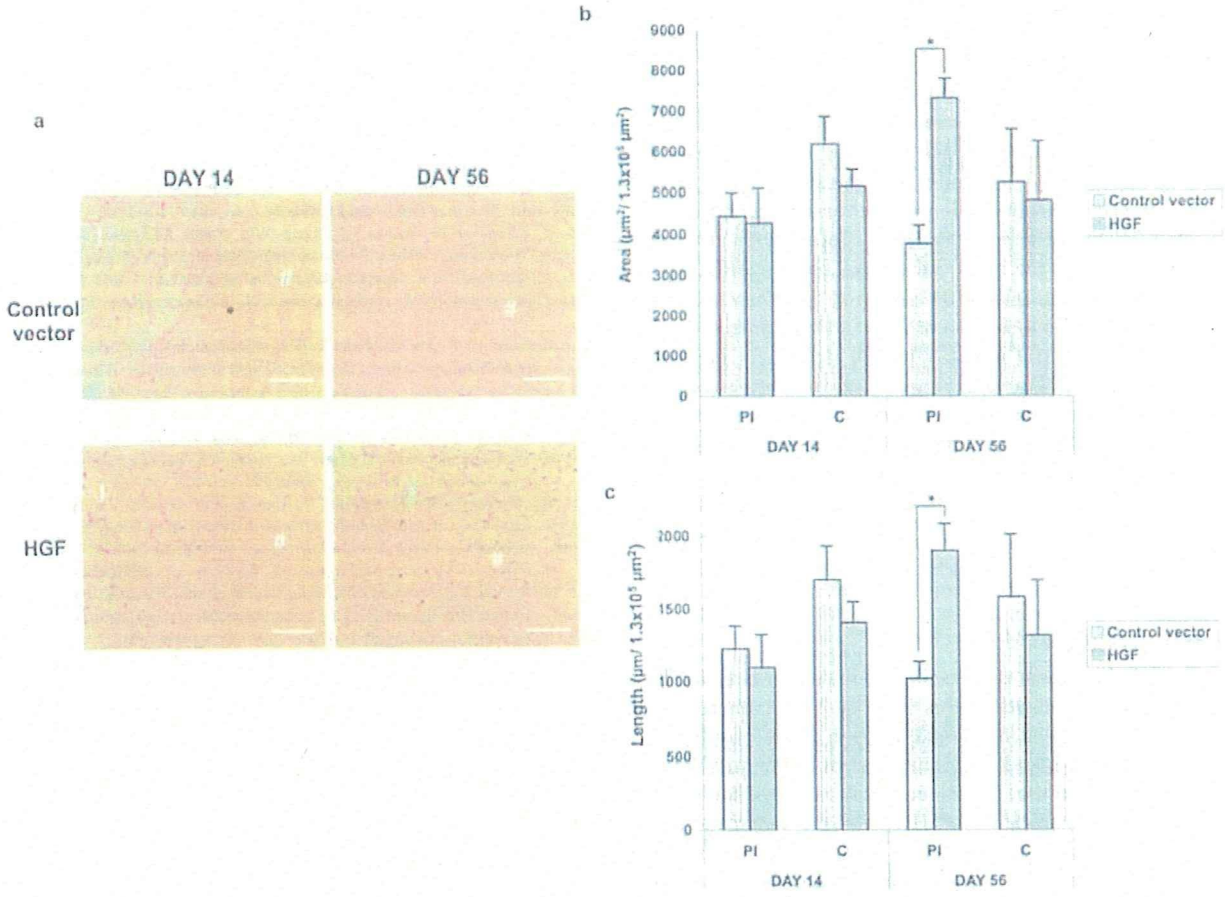


Figure 7. (a) Typical images of ALP staining in ipsilateral neocortex on day 14 in rats transfected with control and HGF vector. Coronal sections of ipsilateral neocortex stained for ALP. The structure of arteries in the peri-infarct region (#) was not different on day 14. However, the arteries in rats transfected with HGF gene showed a more complex pattern on day 56. Bar = 100 μm; I indicates infarct region. (b and c) Quantitative analysis of area (b) and length (c) of blood vessels. PI, peri-infarct region; C, contralateral region; Control vector, rats transfected with control vector (n=4); HGF, rats transfected with HGF vector (n=4). *$P<0.05$ vs Control.

Another possible mechanism is that exogenously added HGF would transiently activate astrocytes and induce the production of other neurotrophic factors, resulting in the promotion of neuritogenesis. In fact, immunoreactivity for GFAP was increased on day 14 but decreased on day 56 to the contrary. Similar results were also observed in the recent report showing the effectiveness of forced arm use and brain-derived neurotrophic factor in MCAo.[35] A recent study showed that the activated astrocytes possess qualities that will promote neuronal survival and regeneration, and they do not, by themselves, produce inhibitory extracellular matrix, whereas reactivated astrocytes stimulated by cytokines, including interleukin 1b, interferon γ, tumor necrosis factor α, and transforming growth factors, contribute to the glial scar formation, which inhibit neuronal survival or regeneration.[37] It was also demonstrated that exogenous HGF regulated c-Met expression in cultured astrocytes and might induce other neurotrophic factors from activated astrocytes.[48] Thus, it is likely that the effect of HGF was direct action and/or indirect action via neuron–glia interactions on neuritogenesis.

This study also revealed an increase in microvessels only in the peri-infarct region but not in normal regions. Although the relationship between the improved microcirculation and behavior is still unclear, a recent report demonstrated that restoration of perfusion by collateral growth and new capillaries in the ischemic border zone around a cortical infarct supported long-term functional recovery in rats.[49] Additionally, others reported that some patients who received tissue plasminogen activator therapy with no immediate clinical improvement in spite of early recanalization showed delayed clinical improvement.[50] From the viewpoints, it is likely that the improvement of microcirculation is an important factor for the functional recovery. Although additional study is necessary, the improvement of microcirculation by HGF might be an alternative mechanism to improve learning and memory.

The influence of HGF on cerebral edema is another important issue. In general, the peak of cerebral edema is 3 days, and a significant decrease is 7 days after permanent MCAo in rats.[51] Afterward, the infarct brain becomes atrophic.[31] In the present study, the infarct region was atrophic in rats transfected with the HGF gene, as well as the control vector, and there was no significant difference in the volume. Thus, HGF gene transfer did not exacerbate the cerebral edema. Considering that VEGF

exacerbated cerebral edema.[51] HGF might be safer than VEGF. Additional study is necessary to compare the effectiveness of HGF to other growth factors.

The amount of HGF produced by this method (0.1 to 0.4 ng/mL) is relatively low because of the limited transfected cells in the surface brain and ischemic region, as compared with that of previous reports showing the effectiveness of recombinant human HGF protein for the cerebral ischemia.[11,23] Nevertheless, this low concentration might be enough to have the beneficial effects, because HGF elicited surviving neurotrophic effect at 0.5 to 1 ng/mL in primary cultured hippocampal neurons[17] and enhanced neurite extension at 0.1 to 100 nM (0.1 to 100 ng/mL) in neocortical explant.[52] Indeed, several previous articles demonstrated that the similar amount of HGF produced by gene transfer showed the neurotrophic and/or angiogenic property in several experimental rodent models.[21,22,25,53] Because the higher concentration of HGF is more effective for survival and neurite extension in in vitro study,[17,52] several improvements, such as modification of the HVJ-envelope vector and HGF plasmid, are required to achieve better outcome.

Perspectives

Overall, the present study is the first to demonstrate that HGF gene therapy delayed for as long as 7 days improved the outcome from ischemic stroke through the reconstitution of the neuronal network and improvement in the microcirculation. In clinical use, the present study might be attractive to support the application of HGF for the treatment of the patients in the chronic stage of brain infarction. Although most of the previous reports demonstrated the effectiveness of growth factors before the insult or within several hours of the onset by the inhibition of apoptosis and extension of the ischemic lesion,[9,10,12,21,22] it is difficult to administer them in time in most patients. Additionally, some patients improve their cognitive dysfunction spontaneously within several days after cerebral infarction. Also, the intracisternal injection is too difficult in the acute stage of cerebral infarction, because it is possible that the brain edema is worsened by intracisternal injection itself. In contrast, the present study is more closed to the real clinical situation for the treatment of the patients with chronic brain stroke. Although additional study is necessary to determine whether other growth factors are effective or not in the chronic stage, gene therapy using HGF may provide new therapeutic options for treatment after cerebral ischemia.

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