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A novel angiogenic method for chronic cerebral hypoperfusion in a rat model

（慢性脳低灌流ラットモデルにおける新たな血管新生法）

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2010年度
A novel angiogenic method for chronic cerebral hypoperfusion in a rat model

Yuki Ohmori

2010年度
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1. Summary

Granulocyte-colony stimulating factor (G-CSF) mobilizes hematopoietic bone marrow cells into systemic circulation and has been used clinically to treat chemotherapy-induced neutropenia. Recently, G-CSF has been shown to have neuroprotective and angiogenetic effects in acute cerebral infarction. We hypothesized that G-CSF could act as an enhancer of angiogenesis after indirect bypass surgery. Chronic cerebral hypoperfusions were induced in male Wistar rats by permanent bilateral internal carotid artery occlusion (BICAO). After BICAO, unilateral indirect bypass and encephalo-galeo-synangiosis (EGS) were performed and human recombinant G-CSF (10 µg/kg) or saline was injected intramuscularly for 5 consecutive days. We measured regional cerebral blood flow (rCBF) by laser Doppler flowmetry and performed immunohistochemical analysis 21 days after BICAO.

BICAO decreased rCBF to 62.52% ± 5.8% of control (P < 0.01). The rCBF increased significantly 21 days after BICAO in all treatment groups (n = 10; P < 0.05) except in the G-E- group. The rCBF increase observed in the G+E+ group was significantly higher than that observed in other groups. Both G-CSF and EGS treatments significantly increased the number of small vessels (P < 0.01), and G-CSF and EGS showed additive effect in increasing the number of small vessels.

Combined use of G-CSF and indirect bypass surgery induces an increase in rCBF and angiogenesis under cerebral chronic hypoperfusion conditions. This is the first report to demonstrate that G-CSF can enhance angiogenesis induced by indirect bypass surgery, and this combined therapy is safe and easy method of treatment.
2. Lists of published papers

1. **Granulocyte-colony stimulating factor enhances the angiogenetic effect of indirect bypass surgery for chronic cerebral hypoperfusion in a rat model**


*Neurosurgery*  in press
3. Acknowledgements

I gratefully acknowledge all of excellent advisers; Dr. Motohiro Morioka, Dr. Takayuki Kawano and Dr. Yasuyuki Kaku (Department of Neurosurgery, Kumamoto University), for the study design and the manuscript preparation. This study has been conducted under the guidance of Professor and Chairman Jun-ichi Kuratsu at Department of Neurosurgery, Kumamoto University Graduate School of Medical Sciences.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Sports, Science and Culture of Japan.
4. Abbreviation and Acronyms

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<td>BICAO</td>
<td>bilateral internal carotid artery occlusion</td>
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<td>EGS</td>
<td>encephalo-galeo-synangiosis</td>
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<td>CPP</td>
<td>cerebral perfusion pressure</td>
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<td>EPCs</td>
<td>endothelial progenitor cells</td>
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<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
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<td>ICA</td>
<td>internal carotid artery</td>
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<td>MCA</td>
<td>middle cerebral artery</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<td>rCBF</td>
<td>regional cerebral blood flow</td>
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<td>SPECT</td>
<td>single photon emission computed tomography</td>
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<td>STA</td>
<td>superficial temporal artery</td>
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<td>TIA</td>
<td>transient ischemic attack</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>vWF</td>
<td>von Willbrand factor</td>
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5. Background and purpose

5-1 The cause of chronic cerebral hypoperfusion

Cerebral hemisphere is received blood supply by 3 major arteries of anterior-, middle- and posterior- cerebral arteries (ACA, MCA, PCA). The largest intracranial artery, internal cerebral artery (ICA) normally supplies to both ACA and MCA (Figure 1A). The sudden and complete occlusion in these arteries by thrombus, induces broad cerebral infarction including frontal-, parietal- and temporal- lobes, because blood supply decreases to lethal level. In these cases, therapeutic time window is very short and effective medical therapy is only clotlysis by tissue plasminogen activator which is only allowed to use within 3 hours from onset because of effectiveness and risk of intracranial hemorrhage. The most cause of the infarction is embolus from extra-cranial region due to the disease, such as arterial fibrillation, deep venous thrombosis and so on. In contrast, the most frequent cause of cerebral infarction is arterial stenosis due to atherosclerosis and so on (Figure 1B). The stenotic lesion shows slow progression and cerebral blood flow decreased slowly. The patient in the early stage of stenosis shows chronic cerebral hypoperfusion and collateral blood flow gradually increases. However the rapid stenosis progression become to beyond collateral vessels ability, the cerebral infarction may occurs (Figure 1B). Even before infarction occurrence, cerebral hypoperfusion may induce brain function impairment such as dementia.

Clinically, this condition is found in diseases like ICA stenosis (or occlusion), middle cerebral artery (MCA) stenosis (or occlusion), moyamoya disease, and so on. Progressive stenosis of the ICA and MCA is mainly induced by atherosclerosis, but the cause of moyamoya disease is still unclear.

5-2 Moyamoya disease

Moyamoya disease is characterized by progressive stenosis of the terminal portion of the bilateral ICA and is associated with an abnormal vascular network, the so-called “moyamoya vessels” (Coyle et al., 1990). In 1997, Research Committee on Spontaneous Occlusion of the circle of Willis (Moyamoya Disease) published guidelines for the diagnosis of moyamoya disease in English (Fukui, 1997). According to these guidelines, moyamoya disease is characterized by stenosis or occlusion at the terminal portions of the ICA or the proximal areas of the anterior or middle cerebral arteries (ACAs,MCAs) and abnormal vascular networks in the arterial territories near the occlusive or stenotic lesions, as shown by cerebral angiography (Figure 1C). The incidence of moyamoya disease is high in countries in east Asia. In Japan, the annual
prevalence and incidence have been estimated at 3.16 and 0.35 per 100 000, respectively. The female to male ratio was shown to be 1.8, and the distribution of age at onset has been suggested to have two peaks: one at 5 years of age and one lower peak at about 25-49 years of age (Wakai et al., 1997). The clinical features of moyamoya disease differ substantially between children and adults. Most children with moyamoya disease develop transient ischemic attack (TIA) or cerebral infarction (Fukui et al., 2000), whereas about half of adult patients develop intracranial bleeding, and half develop TIA or cerebral infarction, or both (Fukui et al., 2000). Data from several studies have shown specific patterns of cerebral hemodynamics in patients with moyamoya disease: pediatric patients have lower cerebral blood flow (CBF) than age matched controls, particularly after ischemic stroke (Ogawa et al., 1990; Kuroda et al., 1993). Cerebrovascular reactivity to acetazolamide is widely impaired in the territory of the ICA, which suggests reduced cerebral perfusion pressure (CPP) (Kuroda et al., 1995).

5-3 Treatment

This chronic hypoperfusion conditions often cause cerebral infarction and require direct bypass surgery (such as superficial temporal artery to MCA anastomosis). Evidence regarding the benefit of direct bypass surgery for patients with a mild decrease of cerebral blood flow (CBF) is controversial (JET Study Group, 2002; The EC-IC bypass study, 1987). For patients with a mild decrease in CBF, there is no treatment except for anti-platelet or anti-coagulant therapy. Patients with moyamoya disease have the option of “indirect bypass surgery,” even in cases of mild CBF decrease. Indirect bypass surgery, which is a relatively easy and safe procedure, can create transgaleal and/or transdural anastomosis from external arteries to a hypoperfused brain via angiogenesis (Figure 2). The effectiveness of this surgery is widely acknowledged in the treatment of moyamoya disease in human (Karasawa et al., 1977; Matsushima et al., 1981; Ozgur et al., 2006; Shirane et al., 1997) and miniature pig (Nakamura et al., 2009); however, in some moyamoya disease patients this treatment fails to provide optimal revascularization from the perspective of collateral formation. Furthermore, for hypoperfused patients with atherosclerotic ICA or MCA stenosis (or occlusion), indirect bypass surgery has a little effect on angiogenesis. As such, a drug that could enhance angiogenesis after indirect bypass surgery and improve the CBF to a hypoperfused brain is needed.

5-4 G-CSF and angiogenesis

G-CSF can mobilize hematopoietic bone marrow cells to enter systemic circulation (Nagata et al., 1986) and has been used clinically to counter the side effects of chemotherapy-induced neutropenia
and prepare donors for peripheral cell harvesting (Morstyn et al., 1989; Neidhart et al., 1989; Sheridan et al., 1989). Recently, G-CSF has been shown to act as a neuroprotective agent in acute cerebral infarction (Morstyn et al., 1989; Gibson et al., 2005; Matchett et al., 2007; Schabitz et al., 2003; Shyu et al., 2006; Six et al., 2003; Solaroglu et al., 2006). Furthermore, several studies have suggested that G-CSF increases collateral angiogenesis after cerebral ischemia (Lee et al., 2005; Piao et al., 2009; Sehara et al., 2007; Toth et al., 2008).

We hypothesized that G-CSF could act as an enhancer of angiogenesis after indirect bypass surgery. In the present study, we investigated the effects of G-CSF on rCBF and angiogenesis after indirect bypass surgery in a rat chronic cerebral hypoperfusion model.
6. Material and Methods

6-1 Animal care and use
All experimental procedures utilized in this investigation were performed in accordance with the institutional guidelines of the Kumamoto University Graduate School of Medical Sciences. Male Wistar rats weighing 300 to 350 g were used in this study. All surgical procedures were performed under anesthesia with 4% halothan and maintained with 1.5% halothan in 70%/30% nitrous oxide/oxygen. Normothermia (37°C) was maintained in each animal utilizing a rectal thermistor probe connected to a feedback-regulated heating blanket.

6-2 Experimental groups
Animals were divided into following 4 experimental groups, the G-CSF injection with EGS treatment group (G+E+), the G-CSF injection without EGS treatment group (G+E-), the saline injection with EGS treatment group (G-E+), and the saline injection without EGS treatment group (G-E-).

6-3 Bilateral ICA occlusion (BICAO) / chronic cerebral hypoperfusion model
Bilateral internal carotid arteries were exposed via a ventral midline cervical incision. A dissection was made between the sternocleidomastoid and the sternohyoid muscles, carefully separated from the sympathetic and vagal nerves. Bilateral internal carotid arteries were doubly ligated with 4-0 silk sutures. (Figure 3A)

6-4 Encephalo-galeo-synangiosis (EGS)
After the BICAO, rats were placed in a stereotactic head holder with the top of the skull positioned horizontally. The scalp was then incised on the parieto-occipital midline, the galea was peeled off the skull, and the galeal flap was prepared. A 7 mm square craniectomy was performed by drilling just behind the coronal suture, 1 mm lateral from the midline, and dural incision was performed with the use of microsurgical forceps and microscope to avoid brain injury. The galeal flap was seated on the region of removed dura. Right-side EGS procedure was performed in the range of 7 mm square just behind the coronal suture, and in the sham-operated animals (the E- group), dural incision was not performed. (Figure 3B)

6-5 Measurement of regional cerebral blood flow (rCBF)
The rCBF was measured before and immediately after and 21 days after BICAO for the ten rats in
each experimental group using transcranial laser Doppler flowmeter (ALF21, Advance, Tokyo, Japan). Details of this procedure have been described previously (Hasegawa et al., 2003). Briefly, rats were anesthetized with halothan and fixed in a stereotactic head holder. A midline scalp skin incision was made and the flowmeter probe was fixed. rCBF was measured 5 times each at the bilateral frontal lobes and cerebellums. We calculated percent values of each frontal rCBF and compared with the ipsilateral rCBF of the cerebellum.

6-6 G-CSF injection
Recombinant human G-CSF was gifted from Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan). Rats were randomly assigned to either the G-CSF (10 µg/kg) or saline injection groups. Injections were administered at the right temporal region intramuscularly. G-CSF (G+ group) or saline (G- group) was injected following carotid artery occlusion once a day for five consecutive days. (Figure 3C)

6-7 Histology and immunohistochemistry
Twenty-one days after BICA0, rats were deeply anesthetized and their aorta were perfused with 300 to 400 ml of phosphate-buffered saline (PBS, pH 7.4), followed by perfusion of 400 ml of 4% paraformaldehyde (PFA) in PBS. Brain tissue (with EGS tissue) was carefully removed, fixed with 4% PFA overnight and embedded in paraffin. From each brain, 5 µm coronal sections including EGS tissue were taken and nonspecific binding sites were blocked in blocking solution (3% bovine serum albumin in PBS). The sections were sequentially incubated overnight at 4°C with an antibody against von Willbrand factor (vWF) (1:200). Immunostaining procedures were carried out using the Vectastain ABC kit to manufacturer instructions, using 3,3’-diaminobenzidine as a chromogen. Adjust sections were Hematoxylin & Eosin (HE) staining for the histological evaluation. Sections were photographed at 40x magnification. Five photographs from various different site in cerebral cortex were taken from each brain section (n=10), and the number and diameter of vWF-positive vessels in each section were recorded. Sections were analyzed blindly by 2 independent co-authors (Y. K. and T. K.) and the mean value was used for final analysis.

6-8 Data analysis
Data are expressed as mean ± standard error (SE). Changes in laser Doppler flowmetry were calculated in percent of mean baseline value. P-values indicate results of a Student’s t-test, and values of P < 0.05 were considered statistically significant.
7. Results

7-1 rCBF change after BICAO in the absence of G-CSF treatment

The relative bilateral frontal lobe rCBFs before and after BICAO were decreased to 55.20 ± 9.27% and 58.24 ± 8.50% in the right and left sides, respectively (P < 0.001, compared to cerebellum rCBF values). There was no statistically-significant difference between the rCBF rates of the right and left sides. Without G-CSF treatment, the rCBFs of the right and left sides increased to 75.21 ± 3.01% and 64.89 ± 3.36%, respectively, 21 days after BICAO. The rCBF rate of the right side (EGS treatment side) was significantly higher than that of the left side (Table 1). Thus, in this hypoperfusion model, a decreased rCBF rate was maintained for 21 days post-BICAO. EGS treatment was associated with a statistically significant increase in rCBF.

7-2 The effect of G-CSF on rCBF

Twenty-one days after operation, increase rate of rCBF of the G+E+, G+E- and G-E+ groups were 91.53%, 59.8%, and 37.0%, respectively, and a with statistical difference was observed when these groups were comparing with the increase rate of 11.4% of G-E- group (rate increase of 11.4%, P < 0.01). The increase rate of rCBF of the G+E+ group was significantly higher than that of the G-E+ group (P < 0.01) or the G+E- group (P < 0.05). Furthermore, there was a significant difference between the increase rates of rCBF the G+E- and G-E+ groups (Figure 4). Thus, G-CSF treatment significantly increases rCBF, and an additive effect is observed when G-CSF is combined with indirect bypass surgery/EGS treatment.

7-3 Immunohistochemical findings of angiogenesis

We performed immunohistochemical analysis of von Willbrand factor (vWF) staining at the transient zone from brain to EGS tissue (galea), using tissues collected 21 days post-BICAO in animals that had been treated with G-CSF (Figure 5 A and B). There was a fuzzy boundary between the cerebral cortex, arachnoid, fibrous coat and galeal tissue on both G+ and G- group received indirect bypass. Numerous vessels smaller than 50 µm were found at the transient zone looks like thin fibrous coat, and extending to the cortex and white matter. There was no brain injury at the site of EGS. (Figure 5 C and F). We counted the vessels number in 100 X 400 µm square at the transient zone and found that the vessel number in G+ group was higher than that of G- group (13.5 ± 4.43 vs 7.7 ± 2.64, respectively: mean ± SD, Figure 5 G and H) with statistical significant difference. Figure 6 shows staining for vWF at sites near the EGS in each experimental group. The G+E+
group had the greatest number of vWF-positive vessels of all treatment groups. The size of these vessels was generally small, with a considerable number under 30 µm.

7-4 Analysis of angiogenesis
G-CSF injection (G+E+ and G+E- group) significantly increased the number of vessels per field, compared with the control G-E- group (G+E+, 65.3 ± 1.8; G+E-, 54.6 ± 2.9; and G-E-, 38.9 ± 2.8; P < 0.01). In the EGS treatment groups (G+E+ and G-E+), G-CSF injection significantly increased the number of vessels per field (G+E+, 65.3 ± 1.8 vs. G-E+.43.9 ± 2.6; P < 0.01). Thus, G-CSF injection has a significant angiogenetic effect regardless of EGS. In the saline injection groups (G-E+ and G-E-), however, EGS treatment did not significantly increase the number of vessels. Thus, although EGS treatment is effective in addition to G-CSF treatment, EGS treatment alone does not significantly impact angiogenesis in this hypoperfusion model (Figure 7).

7-5 G-CSF increases the number of vessels less than 40 µm and modifies the effects of EGS treatment
In addition to number of vessels, we also analyzed the size of proliferated vessels. Figure 8 shows vessel diameter and vessel number at 21 days after operation for each experimental group. Vessels less than 40 µm in size significantly increased in the G+E+ group, compared with the G-E-group (G+E+, 53.8 ± 2.8; G-E-, 28.1 ± 1.9; P < 0.01). In particular, the vessels between 20 to 40 µm significantly increased in the G+E+ group, compared with the G-E+ group (G+E+, 22.7 ± 2.2; G-E+, 13.1 ± 1.4; P < 0.01). The G+E- group also demonstrated a significantly increased number of vessels between 20 and 40 µm, compared with the G+E- group (G+E-, 16.6 ± 0.9; G-E-, 9.7 ± 1.1; P < 0.01). There was not a significant increase in the size of vessels over 40 µm in all groups, however. G-CSF treatment groups (G+E+ and G+E-) had significantly higher numbers of vessels in both the 0-20 µm and 20-40 µm diameter vessel groups, compared with the G- groups.

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8. Discussion

In animal studies examining chronic cerebral hypoperfusion, the bilateral common carotid artery occlusion model is generally used. In this study, we used bilateral internal carotid artery occlusion (BICAO) model. The reasons for this were 1) the BICAO model is more similar to human chronic hypoperfused cerebrovascular diseases, such as moyamoya disease and major cerebral vessel stenosis (or occlusion) than the bilateral common carotid artery occlusion model, and 2) preservation of the blood flow of the external carotid artery was important because we sought to examine angiogenesis after indirect bypass, which creates transcranial anastomosis via the external carotid artery. In contrast to the bilateral common carotid artery occlusion method, which induces severe hypoperfusion (Coyle et al., 1990; Ulrich et al., 1998), there was the possibility with the BICAO method that the decrease in rCBF induced by this method would not be enough to induce experimental hypoperfusion conditions. This could have been due to the development of collateral circulation from the ophthalmic artery and other factors. In our study model, bilateral frontal lobe rCBF decreased mildly, to 50–60 % of that observed in the cerebellum after BICAO (Table 1). As such, our BICAO model mimicks human cerebral hypoperfusion disease quite well.

The main findings of this study were as follows: (1) G-CSF injection and EGS treatment significantly increased rCBF, (2) G-CSF treatment significantly increased rCBF compared to EGS treatment, (3) the effects of rCBF increase induced by G-CSF and EGS were additive, and (4) both G-CSF and EGS significantly increased the number of blood vessels 40 µm or less in diameter. Thus, G-CSF and EGS treatment increased rCBF by inducing angiogenesis, and G-CSF can be considered a powerful angiogenetic enhancer when coupled with indirect bypass (EGS treatment).

The concept of angiogenesis was first described by Folkman et al., who observed the growth of new capillaries during tumor progression (Folkman et al., 1971). Vascular endothelial growth factor (VEGF) was the first angiogenetic factor isolated, and since, several other angiogenetic factors have been found and tested for therapeutic use in the improvement of microcirculation (Gupta et al., 2009). Most studies have utilized single angiogenetic agents for the treatment of cerebral acute/chronic hypoperfusion conditions; there is a lack of studies focusing on combination therapy utilizing angiogenetic agents and indirect bypass surgery. Kusaka et al. injected plasmids bearing the human VEGF gene into rats following indirect bypass for chronic cerebral hypoperfusion and reported a remarkable increase in capillary number following the VEGF injection (Kusaka et al., 2005). Our study is the second report of a combination therapy that includes angiogenetic factor treatment and indirect bypass surgery and demonstrates significant
increases in rCBF and vessel number. While most therapeutic trials utilizing angiogenic factors involve gene therapy, our study utilized G-CSF, a protein that is safe and widely used in humans.

G-CSF is known to have neuroprotective and neurogenesis activities in acute cerebral ischemia in experimental animals (Gibson et al., 2005; Matchett et al., 2007; Schabitz et al., 2003; Six et al., 2003; Solaroglu et al., 2006; Sevimli et al., 2009) and humans (Shyu et al., 2006). Recently, it was reported that G-CSF has angiogenic activity when administrated after cerebral infarction (Lee et al., 2005; Piao et al., 2009; Sehara et al., 2007; Toth et al., 2008). The mechanism of angiogenesis by the G-CSF is not well known. G-CSF mobilizes CD34+ hematopoietic stem cells (HSCs) from the bone marrow into peripheral blood (Grigg et al., 1995). It has been suggested that bone marrow-derived endothelial progenitor cells (EPCs) participate in cerebral neovascularization after cerebral ischemia (Taguchi et al., 2004; Zhang et al., 2002). Because bone marrow-derived CD34+ cells contain EPCs (Asahara et al., 1997), EPCs mediating the angiogenesis induced by G-CSF are very likely to originate in the bone marrow (Natori et al., 2002). Furthermore, G-CSF activates endothelial cell proliferation in vitro and demonstrates angiogenic activity in the corneal angiogenesis assay (Bussolino et al., 1991). Ohki et al. reported that VEGF was released by neutrophils that had been prestimulated with G-CSF in vitro and in vivo, which suggests that G-CSF may stimulate the VEGF-mediated angiogenesis system (Ohki et al., 2005). Thus, G-CSF may contribute to several pathways of angiogenesis in ischemic or hypoperfusion conditions.

In moyamoya disease, direct bypass (STA-MCA anastomosis) is considered more effective than indirect bypass. However, in some patients (especially pediatric patients) direct STA-MCA bypass can be difficult, due to both the size and progressive occlusion of the MCAs. In contrast, indirect bypass and cerebral revascularization has been shown to be beneficial in both younger patients (Houkin et al., 1997; Ishikawa et al., 1997; Lee et al., 2003; Matsushima et al., 1991) and adult patients (Houkin et al., 1997; Han et al., 1997; Starke et al., 2009). Although indirect bypass is considered a beneficial surgery for most moyamoya disease patients, indirect bypass surgery fails to provide satisfactory revascularization in some patients. As such, the addition of G-CSF treatment would be a powerful enhancer for angiogenesis in cases in which surgery alone proved unsatisfactory.

In contrast to its use in the treatment of moyamoya disease, indirect bypass does not appear to be beneficial in patients with medically refractory symptomatic intracranial athero-occlusive disease and secondary hemodynamic failure (Komatar et al., 2009). This has been suggested to be owing to the fact that patients with atherosclerotic disease may have impaired
angiogenesis secondary to reduced endothelial repair capacity. For example, EPCs (which have been characterized as KDR+/CD133+ cells) are reduced in patients with atherosclerotic risk factors and cardiovascular disease (Asahara et al., 1997; Hill et al., 2003). Rafat et al. reported that circulating EPCs increase in moyamoya disease patients and may play a role in angiogenesis (Rafat et al., 2009). They also found that the level of circulating EPCs in atherosclerotic cerebro-vascular disease patients was higher than that of healthy controls, but lower than that of moyamoya disease patients. Furthermore, G-CSF treatment has been demonstrated to increase the number of EPCs in patients with myocardial infarction (Bruno et al., 2006). The combination of this data and our data suggests that combination therapy consisting of G-CSF treatment and indirect bypass surgery could increase circulating EPCs, which could induce angiogenesis in patients with atherosclerotic hypoperfusion conditions.
9. Conclusion

The present study is the first to report that G-CSF treatment stimulates angiogenesis of intraparenchymal microvessels and enhances angiogenesis at EGS sites in a model of cerebral hypoperfusion. G-CSF and EGS treatment increases cerebral blood flow and induces angiogenesis in a rat chronic cerebral hypoperfusion model. Moreover, G-CSF enhances angiogenesis associated with indirect bypass surgery. Although G-CSF has clinically some side effects such as bone pain, fever and allergic reaction, most of them are rare and transient (Morstyn et al., 1989). Thus G-SCF is considered as a highly safe drug and used for many patients now. Our results imply that G-CSF treatment combined with indirect bypass surgery could become a novel method for treating chronic cerebral hypoperfusion.
10. References


11. Figure and Figure legends

Table 1.
Changes in rCBF measured by transcranial laser Doppler flowmeter pre-/post-BICAO and 21 days after BICAO. Mean rCBF values from 10 animals per group were calculated. rCBF was measured 5 times in each animal, and the percent value compared to rCBF measured at same side of the cerebellum.

Figure 1.
Representative cases of normal cerebral perfusion, MCA stenosis induced by atherosclerosis and moyamoya disease.
A : Normal cerebral angiography and scheme, it shows internal cerebral artery (ICA) normally supplies to both ACA and MCA
B : Cerebral angiography and scheme show MCA stenosis due to atherosclerosis. The lesion causes the small infarction on Magnetic Resonance Imaging (MRI).
Moreover the stenotic lesion induces cerebral blood flow decrease on N-isopropyl-p-(123)I iodoamphetamine ((123)I-IMP) single photon emission computed tomography (SPECT).
C : moyamoya disease is characterized by stenosis or occlusion at the terminal portions of the ICA or the proximal areas of the anterior or middle cerebral arteries (ACAs, MCAs) and abnormal vascular networks in the arterial territories near the occlusive or stenotic lesions, as shown by cerebral angiography and scheme. SPECT shows cerebral hypoperfusion induced by progressive arterial stenosis.

Figure 2.
The scheme of the clinical direct and indirect bypass surgery.
Microscopic view of direct bypass (superficial temporal artery – middle cerebral artery anastomosis) and indirect bypass (encephalo-duro-galeo-synangiosis).

Figure 3.
The scheme shows the procedures of bilateral ICA occlusion (A), encephalo-galeo-synangiosis (B) and the schedule of experimental procedures (C).

Figure 4.
Percent rCBF increase at 21 days after BICAO in each treatment group.
G: G-CSF treatment; E: EGS treatment; **, $P < 0.01$, compared with G-E- group; ††, $P < 0.01$, compared with G+E+ group; †, $P < 0.05$, compared with G-E+ group.

Figure 5.
H&E staining of whole brain in each G-CSF (+) and G-CSF (-) group received EGS (A and B) Elastic van Gieson staining (B and E) and Immunohistochemical staining for von Willbrand factor (C and F) at the transient zone between brain (lower side) and galeal tissue of EGS (upper side). Scale bar shows 100 µm. We counted the vessels number in this transient zone, not brain, in 100 X 400 µm square (G). The vertical bar showed the mean vessel number in each group (13.5 ± 4.43 and 7.7 ± 2.64, respectively: mean ± SD, n=10)

Figure 6.
Representative immunohistochemical staining for von Willbrand factor in brain sections from different three animals for each treatment group. G+E+ group (A, B, C), G+E- group (D, E, F), G-E+ group (G, H,I), G-E- control group (J,K,L). Scale bar shows 100 µm.

Figure 7.
Vessel number for each treatment group 21 days after BICAO. **, $P < 0.01$, compared with G-E- group; ††, $P < 0.01$, compared with G-E+ group; †, $P < 0.05$, compared with G-E+ group; *, $P < 0.01$ compared with G+E- group.

Figure 8.
Vessel number distribution for each vessel diameter size group. **, $P < 0.01$, compared with G-E- group; ††, $P < 0.01$, compared with G+E+ group; †, $P < 0.05$, compared with G-E+ group.
Table 1

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<th>% rCBF compared to cerebellum (mean ± SE)</th>
<th>P-value</th>
<th>Rt vs.Lt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rt side (EGS+)</td>
<td>Lt side (EGS-)</td>
<td></td>
</tr>
<tr>
<td>0 day/ pre-BICAO</td>
<td>95.34 ± 7.42</td>
<td>99.68 ± 7.82</td>
<td>N.S.</td>
</tr>
<tr>
<td>0 day/ post-BICAO</td>
<td>55.20 ± 9.27</td>
<td>58.24 ± 8.50</td>
<td>N.S.</td>
</tr>
<tr>
<td>21 day post-BICAO</td>
<td>75.21 ± 3.01</td>
<td>64.89 ± 3.36</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>* %</td>
<td>136.25 ± 5.45</td>
<td>111.41 ± 5.77</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

BICAO, bilateral internal carotid artery occlusion; EGS, encephalo-galea-synangiosis; rCBF, regional cerebral blood flow

* % of rCBF 21days after BICAO, compared with rCBF value 0 days after BICAO
Figure 1

A Normal

B Atherosclerosis

C Moyamoya disease
Figure 2

Direct bypass (STA-MCA)

Indirect bypass (EDGS)
ICA occlusion

vagus

7mm

EGS

Day 1

Day 5

Day 21

Perfusion & fixation

G-CSF or Saline injection
(10µg/kg)

BICAO

EGS

28
Figure 4

% increase of rCBF (%)

** P<0.01  VS. G-E- group
n = 10 / group

†† P<0.01

† P<0.05
Figure 5

A. G-CSF (+)

D. G-CSF (-)

G. 400 X 100 µm

H. Vessels/square

B and C: Images showing the brain tissue with G-CSF (+) and Galea

E and F: Images showing the brain tissue with G-CSF (-) and Galea

H: Bar graph showing the comparison of vessels/square between G-CSF (+) and G-CSF (-) with a significance level of P<0.01.
Figure 6

A  D  G  J
B  E  H  K
C  F  I  L

G+E+  G+E-  G-E+  G-E-
Figure 7

Vessels/field

G+E+

G+E-

G-E+

G-E-

** P<0.01 VS G-E- group
n = 10 / group

* P<0.01

** P<0.05

† P<0.001
Figure 8

* P < 0.05 vs G-E- group
** P < 0.01 vs G-E- group

n = 10 / group

vessels/field

vessel diameter (μm)

G+E+  G+E-  G-E+  G-E-

† P < 0.05
‡‡ P < 0.01