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G-CSF Mobilized Peripheral Blood Human Hematopoietic CD34+ Stem Cells Therapy for Acute Stroke: Preliminary Results

[1]Department of Neurosurgery, Cerebrovascular Center, Yongdong Severance Hospital, Yonsei University College of Medicine, Seoul, Korea
[2]Department of Neurosurgery, Pundang CHA Hospital, Pochon CHA University, Seongnam, Korea
[3]Cell & Gene Therapy Research Institute, College of Medicine, Pochon CHA University, Seoul, Korea
Jung Yong Ahn, MD[1] · In Bo Han, MD[2] · Dong Ku Kim, PhD[3]

ABSTRACT

Objective : Acute stroke caused by cerebral artery occlusion or rupture is the most important vascular central nervous system disorder in Korea and remains a leading cause of death and disability despite significant clinical benefits after current treatment modalities. Therefore, it is crucial to develop new alternative therapeutic strategies. The most encouraging approach is directed towards cell transplantation into damaged regions. We discuss the ideal candidate for cell transplantation in current status and preliminary results of peripheral blood stem cells transplantation for acute stroke. **Methods** : Five patients with acute stroke (three patients with deep intracerebral hemorrhage and two patients with middle cerebral artery occlusion) underwent peripheral blood stem cells transplantation stereotactically. **Results** : Cell transplantation of three patients did not improve motor function recovery, as evidenced by NIHSS. However, interestingly, cell transplantation significantly increased CSF levels of vascular endothelial growth factor (VEGF). **Conclusion** : Cell transplantation did correlate positively with elevated growth factor levels in CSF, but not with improved motor function. **(Kor J Cerebrovascular Surgery 8:235-40, 2006)**

KEY WORDS : Acute stroke · Cell transplantation · Hematopoietic stem cells · CD34 · Vascular endothelial growth factor.

Introduction

The delayed, ineffective management of acute ischemic stroke result in high rates of morbidity and mortality. For patients with acute ischemic stroke who present with serious neurologic symptoms on admission or continue to deteriorate neurologically due to total occlusion despite maximal medical treatment, an effective intervention to improve their neurologic symptoms and clinical outcome has not yet been established.

Bone marrow (BM) or mobilized peripheral blood-driven stem cell implants have been reported to regenerate damaged brain tissues.[7)19)30)] BM-derived stem cells have been shown to differentiate in *in vitro* cultures into neurons.[7)] Similarly, they were reported to regenerate ischemic brain *in vivo*.[30)] Data obtained on human CD34+ BM cells, show that systemically administered CD34+ cells migrate to the site of damage where they contribute to healing in the rat.[8)] These observations aroused many hopes for the development of new therapeutic strategies to ameliorate neurological deficits in patients after stroke.

The potential role of BM-derived progenitor/stem cells in the regeneration of damaged tissue, however, is not clear at this point. Generally, the beneficial effect of these cells in regeneration could be explained by (1) trans-

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교신저자 : Dong Ku Kim, PhD, Cell and Gene Therapy Research Institute, College of Medicine, Pochon CHA University, 605, Yeoksam 1-dong, Kangnam-gu, Seoul, 135-081, Republic of Korea
전화 : (02) 3468-3394 • 전송 : (02) 3468-3264
E-mail : dokukim@hanmail.net
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dedifferentiation/plasticity of hematopoietic stem cells,[3)9)17)] (2) paracrine secretion of angiopoietic factors from BM-derived stem/progenitor cells which improve vascularization of the damaged organ, or (3) the presence of a heterogeneous population of tissue-committed stem cells in the BM.[14)]

The progenitor/stem-enriched CD34+ cells could be mobilized into peripheral blood by administration of growth factors (e.g. G-CSF), or chemotherapeutics (e.g. cyclophosphamide), or a combination of them.[5)23)] The expression of the CD34 surface antigen characterizes a heterogeneous population of cells including hematopoietic progenitor cells (HPCs), endothelial progenitor cells (EPCs), and mature endothelial cells. Although the true role of the CD34 molecule continues to be debated, CD34+ HSPC have been functionally defined as capable of generating progenitor-derived clones *in vitro* and by their potential in reconstituting the lymphomyelopoietic system in myelocompromised hosts.[4)18)28)] There are no data so far on mobilization of these cells after ischemic stroke.

The aim of this study was to evaluate the short-term efficiency afte stereotactically injection of G-CSF-mobilized hematopoietic CD34+ stem cells on peripheral bood in patients with ischemic or hemorrhagic strokes with severe neurological deficits. Additionally, we assessed VEGF levels in CSF for the neurogenic and angiogenic capacity after the stroke attack.

Patients and Methods

Patients

Approval was granted from our institutional review board to study hematopoietic CD34+ stem cells transplantation as a treatment for acute stroke with severe disabilities. From June 2004 to June 2005, we identified 5 patients who underwent G-CSF-mobilized hematopoietic CD34+ stem cells transplantation (Table 1). Two patients with acute middle cerebral artery (MCA) occlusion were eligible for the study if they had the following characteristics: (1) they had been observed within 7 days of the onset of symptoms; (2) they were relevant lesions within the MCA territory as assessed using diffusion-weighted imaging; and (3) they had experienced severely disabling deficits that persisted for longer than 7 days (according to the National Institutes of Health Stroke Scale [NIHSS], a score of 7 or more points after 7 days of admission is severe) regardless the conventional stroke treatment. Three patients with deep intracerebral hemorrhage were eligible for the study if they had the following characteristics: (1) they had severe neurologic motor deficit (NIH motor scale, a score of 2 or less points is severe); (2) they were relevant lesions with non-visualization of posterior internal capsule (PIC) as assessed using diffusion-tensor imaging; and (3) they had experienced severely disabling deficits that persisted for longer than 5 days regardless the stereotactic evacuation of the hemorrhage. We excluded patients who met one of the following criteria: hematological cause of stroke, severe co-mobidity, hepatic or renal dysfunction, or unwillingness to participate. Informed consent was obtained from patients or medical guardians.

Mobilization and Collection of peripheral blood hematopoietic stem cells

10 μg/kg of recombinant human granulocyte-colony-stimulating factor (G-CSF) was administered subcutaneously every 24 hours for 5 days. Since the previous study on subcutaneous administration of G-CSF, peripheral blood CD34+ levels reached near maximum after 4 days of G-CSF treatment,[19)] we colleted peripheral blood stem cells on the

Table 1. Summary of characteristics of patients with G-CSF mobilized peripheral blood hematopoietic CD34+ cells transplantation

Case	Age(yrs)/ Sex	Stroke	Location of lesion	NIHSS at admission	NIHSS 14 days posttransplantation	NIHSS 1 month posttransplantation	NIHSS 6 months posttransplantation
1	62/F	Ischemic	MCA territory	18	16	15	14
2	58/F	Hemorrhagic	Thalamus	14	14	13	14
3	64/M	Hemorrhagic	Thalamus	15	12	10	7
4	70/M	Ischemic	MCA territory	19	17	17	15
5	55/F	Hemorrhagic	Thalamus	16	15	13	12

G-CSF: Granulocyte-colony stimulating factor, NIHSS: National Institutes of Health Stroke Scale, MCA: middle cerebral artery

day following the fourth dose of G-CSF. The total blood volume processed per apheresis was three times the donor's blood volume at flow rates of 70~80 ml/min. Whole blood: anticoagulant ratio was programmed and anticoagulant citrate dextrose solution (ACD-A) was used as an anticoagulant. Total $CD34^+$ cells were assayed according to the International Society of Hematotherapy and Graft Engineering (ISHAGE) guideline.[21] The target dose of $CD34^+$ cell/kg was a maximum of 4×10^6/kg of patients. Daily collection was performed to reach the target dose.

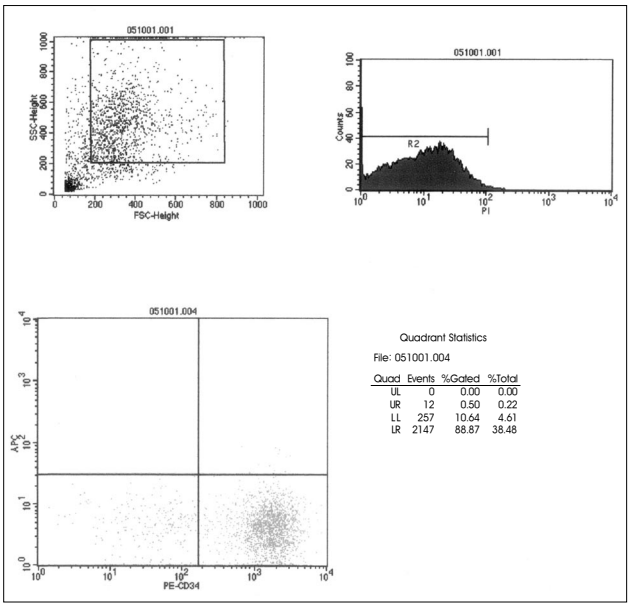

Fig. 1. Representative study of flow-cytometry measurements of the number of $CD34^+$ cells in peripheral blood by use of fluorescein isothiocyanate-conjugated anti-CD34 monoclonal antibodies.

Immunophenotyping

Peripheral blood mononuclear cells (MNC) were isolated by standard Ficoll-Hypaque (Amersham Biosciences, Uppsala, Sweden; density, 1.077 g/ml) density gradient centrifugation. Isolated cell preparations (1×10^6 MNC) were incubated for 30 min at 4℃ in the dark with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 (HPCA-2; Becton Dickinson, San Jose, CA, USA). As isotype controls for the staining, we used FITC-conjugated mouse immunoglobulin G1. Phenotypic analysis of $CD34^+$ cells was performed with a FACS Calibur flow cytometer (Becton Dickinson, Rutherford, NJ) equipped with a 15-mW air-cooled argon-ion laser tuned at 488nm. Cell Quest software was used for data acquisition and analysis (Fig. 1).

Cells transplantation

On the day of injection, a MRI with axial slices of 5 mm for calculation of the trajectory was performed. Targets were located at the ischemic penumbra zone or perihematoma zone (Fig. 2). Patients were then transferred to the operating room and were placed on the Leksell stereotactic frame (Elekta Instruments, Stockholm, Sweden). Catheter was stereotactically inserted to target site. 5×10^6 cells were injected to the target sites.

Measurement of improvements

The NIHSS score as an index of neurological deficit were administered at regular intervals for up to 6 months after the cell transplantation. Serial MRI was performed at 1 month and 6 months after the cell transplantation. For assessing VEGF levels in CSF, we obtained CSF by lumbar tab at

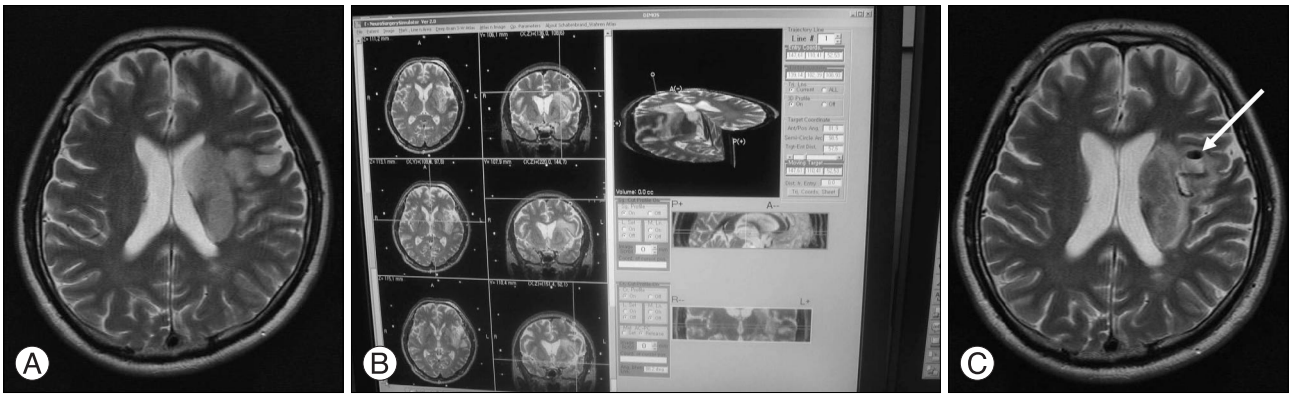

Fig. 2. Case 1 with acute middle cerebral artery occlusion. A : Pretransplantation T2-weighted axial image. B : Localization of target sites and calculation of the trajectory. C : Posttransplantation T2-weighted axial image. Arrow means the transplanted target site.

regular intervals (every week) for up to 1 month. ELISA for VEGF was performed using commercially available kits R & D Systems.

Statistical analysis

Difference between the final NIHSS score and the final VEGF concentration in CSF was examined using Fisher's exact test. Statistical significance was established at $p<0.05$.

Results

Clinical, laborato

y, radiographic evaluations of the G-CSF mobilized hematopoietic CD34+ stem cells-treated patients showed no deaths, stroke recurrence, or cell-related serious adverse reactions. There was no immediate or delayed toxicity related to stereotactic injection of stem cells during the follow-up periods.

The mean NIHSS score was 16.4 ± 2.1 at admission, 14.8 ± 1.9 at 14 days posttransplantation, 13.6 ± 2.6 at 1 month posttransplantation, and 12.4 ± 3.2 at 6 months posttransplantation, respectively (Fig. 3). The VEGF concentration in CSF was 4.6 ± 2.4 pg/ml as basal levels, 24.0 ± 6.7 pg/ml at 7 days posttransplantation, 48.2 ± 17.2 pg/ml at 14 days posttransplantation, 49.0 ± 15.6 pg/ml at 21 days posttransplantation, and 58.4 ± 17.3 pg/ml at 28 days posttransplantation, respectively (Fig. 4). Difference between the final NIHSS score and the final VEGF concentration in CSF was significant (p value=0.048).

Discussion

This present data provide the first evidence that increased VEGF concentration in CSF is strongly correlated with improved neurological outcome in patients with with G-CSF mobilized peripheral blood hematopoietic CD34+ cells transplantation. However, it is controversial whether spontaneous elevation of VEGF concentration after stroke attack or stimulating effect of exogenous stem cells transplantation was the cause for these unexpected outcomes.

Both global forebrain ischemia and focal ischemia, or MCA occlusion, have been shown to induce neurogenesis in the dentate gyrus in adult mammals.[1)6)29)] Studies in intact animals provide evidence that various molecules can enhance the intrinsic neurogenic capacity and thus might be suitable to promote neuronal replacement following ischemic events in the CNS. Vascular endothelial growth factor (VEGF) elicits a delayed response on neurogenesis following MCA occlusion in rats, which is attributed to a survival rather than a proliferation effect.[20)] Reduction o infarct volume and functional recovery point to an additional neuroprotective effect exerted by VEGF. Interestingly, brain-derived neurotrophic factor (BDNF), which enhances neurogenesis in intact animals, decreases neurogenesis in a global ischemia model following adeno-associated virus-mediated BDNF overexpression in the hippocampus.[10)] Further analysis indicates that these effects are at least

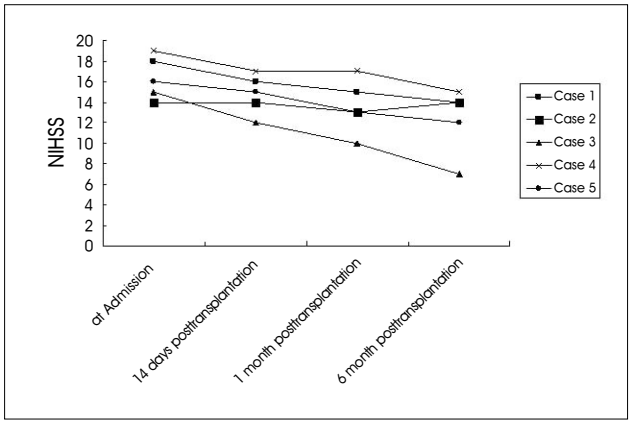

Fig. 3. The National Institutes of Health Stroke Scales of five patients with G-CSF mobilized peripheral blood hematopoietic CD34+ cells transplantation.

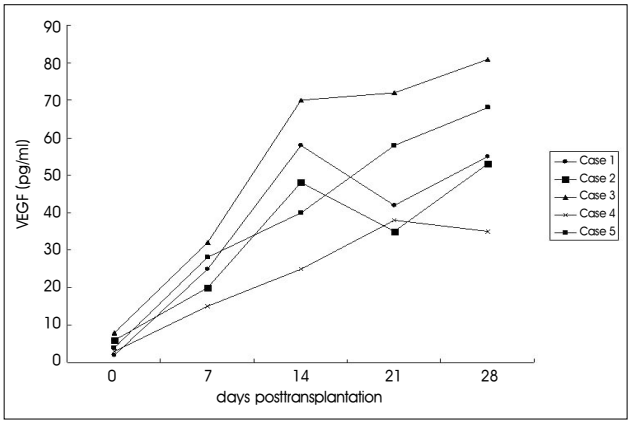

Fig. 4. VEGF concentration in cerebrospinal fluid of five patients with G-CSF mobilized peripheral blood hematopoietic CD34+ cells transplantation.

partially mediated through upregulation of VEGF and BDNF expression. None of the above-mentioned experiments can clearly attribute the observed functional benefit to the therapeutic agents induced effects on neurogenesis.

The rationale to transplant stem cells into the damaged brain is primarily to reconstruct the destroyed neuronal circuit. In addition, cell grafts may exert a neuroprotective effect by secreting survival promoting neurotrophic factors either intrinsically or after introduction of therapeutic transgenes. Very few clinical data are available regarding adult stem cells transplantation in stroke patients. Only one clinical study investigated the effects of autologous mesenchymal stem cell (MSC) transplantation in patients with severe cerebral infarcts.[2] Serial evaluations showed no adverse cell-related, serological, or imaging-defined effects. The intravenous infusion of autologous MSCs appears to be a feasible and safe therapy that may improve functional recovery. However, the fate of the transplanted MSCs is unknown; there have been rarely reported about MSCs related to tumorigenesis.[16][25]

The discovery that hematopoietic stem cells are capable of transdifferentiating into neural lineages,[13] together with the accessibility of hematopoietic stem cells has shifted the focus of attention into the application of hematopoietic stem cells as a promising cell replacement strategy in the diseased CNS. Subsequently, convincing evidence accumulated, which questioned the occurrence of transdifferentiation. Recent studies discovered that the phenomenon of cell fusion and procedural artifacts in determining cell fate account for the expression of neural lineage markers rather than true transdifferentiation.[11][24][27] Nevertheless, a vast number of preclinical studies were conducted investigating the regenerative capacity of hematopoietic stem cells following brain damage either by mobilization of intrinsic pools or by transplantation of hematopoietic stem cells.[15]

Various sources of hematopoietic stem cells have been used for preclinical studies: bone marrow stromal cells, umbilical cord blood cells and G-CSF mobilized peripheral blood cells ($CD34^+$ cells). The number of hematopoietic stem cells in the peripheral blood can be increased by mobilizing cells from the bone marrow using G-CSF, thus serving as an effective source for autologous hematopoietic stem cells transplantation. Isolating hematopoietic stem cells from peripheral blood can be performed without risk or potential side effects. The first preclinical data suggest that peripheral blood derived hematopoietic stem cells are as efficient as umbilical cord blood derived hematopoietic stem cells in promoting functional improvement following MCA occlusion and intravenous transplantation in rats.[26]

We are aware that at this point it is still premature to speculate on the biological function of mobilized hematopoietic stem cells for acute stroke. First, these cells may accompany mobilization of other more primitive hematopoietic and non-hematopoietic stem cells (e.g. endothelial, neural stem cells) that are mobilized into peripheral blood in an attempt to regenerate brain directly. Secondary, hematopoietic $CD34^+$ stem cells are a rich source of several proangiopoietic factors.[12] Thus, it is possible that they may home to the damaged tissue and by secreting aniopoietic factors contribute to neovascularization. These possibilities will be tested in our investigations.

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