ONLINE SUPPLEMENT

SUPPLEMENTAL METHODS

Supplemental Methods

BM Transplantation

BM transplantation was performed as previously described.[1] BM cells ($2X10^6$) from 8-week-old donor mice were injected into lethally irradiated (9.7 Gy from GC3000 Irradiator, MDS-Nordion, Ottawa, Canada) recipient mice via the tail vein. To facilitate the determination of the reconstitute rate, we transplanted GFP positive BM cells to a group of WT mice in parallel. Circulating blood was collected 4 weeks after BM transplantation. Flow cytometric analysis showed that 90-93% of nucleated cells in the recipient mice expressed GFP, indicating a high reconstitute rate of recipient BM by donor BM**.**

WBC Quantification and Sample Collection

Four weeks after the BM transplantation, recipient mice were anesthetized using ketamine/xylazine (100/10 mg/kg body weight). Two μl of viral suspension containing 2 X 10^9 genome copies of AAV-VEGF or AAV-LacZ were injected into the right basal ganglia at a rate of 0.2 μl per minute.

Circulating blood was collected from the retro-orbital plexus on days 0, 5, 8, 11, 14, 17, 21, and 25 after gene transfer, and WBCs were quantified with a HEMAVET (Model 950, DREW Scientific Inc, Oxford, CT). All blood samples were taken at the same time of the day. A total of 50 μl of blood was collected from each mouse at each time point for the measurement. Mice were sacrificed 4 weeks after vector injection, and the brain and femur were harvested. Before the animals were euthanized, BrdU (100 mg per mouse) was injected intraperitoneally to the mice daily for 5 consecutive days.[2]

AAV Vector Injection

Four weeks after the BM transplantation, recipient mice were anesthetized using ketamine/xylazine (100/10 mg/kg body weight). Two μl of viral suspension containing 2 X 10^9 genome copies of AAV-VEGF or AAV-LacZ were injected into the right basal ganglia at a rate of 0.2 μl per minute.

Immunohistochemistry

Twenty μm thick brain coronal cryostat sections, prepared from 1 mm rostral to 1 mm caudal of the injection site, were used for immunohistochemical staining. Antibodies specific to CD45 (BD Biosciences, San Jose, CA), BrdU (Sigma-Aldrich, St. Louis, MO), CD31 (BD Biosciences), and MMP-9 (a gift from Dr. Robert Senior of Washington University, St. Louis, MO) were used.

Quantification of Microvessel Density

We have established a relatively simple and reliable method to quantify the number of microvessels in a given brain region using lectin (fluoresceinlycopersicin esculentum lectin, Vector Lab)-stained sections, referred to as "microvessel density."[3] The microvessel density is heterogeneous in the cerebral cortex by both region and cortical layer. Thus, we injected the viral vector into the basal ganglia and analyzed the microvessel density in the pictures taken from similar regions in all the animals. Briefly, the injection needle was inserted in a burr hole drilled into the pericranium 1 mm lateral to the sagittal suture, 1 mm posterior to the coronal suture, and 3 mm under the cortex surface. We took 2 sections, 1 mm rostral and 1 mm caudal of the injection site, for the microvessel density analysis. Sections were stained with lectin (2 μg/ml, Vector Laboratories). Three images were taken from each lectin-stained section (to the right and left of and below the injection site) under 10X objective lens (Figure 4A & B). Microvessel numbers in each picture were counted using NIH Image 1.63 software. Two investigators without knowledge of treatment conditions assessed vessel counts separately. Microvessel density for each animal was calculated as the mean of the microvessel counts obtained from the six pictures. The microvessel density analyzed in the sections selected as described above should be comparable.

Gelatin Zymogram for MMP-9 Activity Detection

Equal amounts of protein prepared from brain tissues were loaded onto a Novex 10% gelatin gel (Invitrogen). Gel was subjected to electrophoresis and stained with Coomassie Blue R-250 (Bio-Rad Laboratories, Hercules, CA). The densities of MMP-9 bands were quantified using NIH Image 1.63 software. The data are presented as the densities of each sample relative to the mean density of MMP-9 KO+KO BM group.

Statistical Analysis

Data are expressed as mean±SD. The influence of VEGF on the WBC counts, microvessel density, and MMP-9 activity was analyzed using one-way ANOVA followed by Fisher's protected least significant difference (PLSD) test. A probability (P) value <0.05 was considered statistically significant.

We also analyzed the correlation of microvessel densities with recipient genotype (MMP-9 KO or WT), donor BM genotype (MMP-9 KO or WT) and injection type (AAV-VEGF or AAV-LacZ) using a three-way ANOVA analysis. To estimate the effect magnitude, we performed linear regression assay.

References

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