ORIGINAL CONTRIBUTION

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The early administration of granulocyte colony-stimulating factor increases the engraftment of transplanted bone marrow-derived cells into the olfactory epithelium damaged by methimazole*

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SUMMARY

In 2005 we reported that transplanted bone marrow-derived cells were engrafted into the olfactory epithelium and then had characteristics of olfactory neuron cells. However, the engraftment rate was far from a practical level. Granulocyte colony-stimulating factor (G-CSF) is known to mobilize stem cells from the bone marrow into the circulation. To assess the efficacy of G-CSF on engrafting transplanted bone marrow-derived cells into the olfactory epithelium, we performed the time window study of G-CSF administration. After C57BL/6 mice transplanted with bone marrow cells from GFP mice received selective damage of the olfactory epithelium by methimazole, G-CSF was administrated into the recipient mice at different time windows. A statistical analysis demonstrated that the early administration of G-CFS was appropriate to increase the engraftment rate of bone marrow-derived cells into the olfactory epithelium. Cells with double-immunostaining for GFP and OMP, GAP43 or cytokeratin were found in the olfactory epithelium of recipient mice. These results suggest that bone marrow-derived cells are engrafted as stem cells of the olfactory tissue and that the early administration of G-CSF is more effective to increase the engraftment rate of bone marrow-derived cell into the olfactory epithelium damaged by methimazole.

Key words: bone marrow cells, engraftment, G-CFS, methimazole, olfactory tissue, transplantation

INTRODUCTION

Bone marrow stem cells are known to have plasticity of differentiation beyond cell lineages [1-7]. We reported that donor bone marrow-derived cells were engrafted in the olfactory epithelium of radiation-induced immunotolerant recipient mice and then a subpopulation had obtained characteristics of olfactory neuron cells [8]. This report raised two problems to be resolved for recovery of any olfactory function. The first problem was the low rate of engraftment of bone marrow-derived cells into the olfactory epithelium and the second problem was synaptogenesis between olfactory neurons and mitral and tufted cells in the glomeruli of the olfactory bulb. Apart from synaptogenesis, the engraftment rate in those experiments was only 0.1%, partly explained by the possibility that the olfactory epithelium was not injured except for radiation. This engraftment rate was not sufficient for clinical adaptation to olfactory regeneration.

Because granulocyte colony-stimulating factor (G-CSF) can mobilize stem cells from the bone marrow into the circulation, G-CSF is used clinically to cope with neutropenia after cancer chemotherapy and to secure bone marrow transplantation [9]. Circulating mobilized stem cells can be recruited into the damaged tissue and this homing behavior is considered to be one of repair mechanisms in pathological conditions [10,11]. The administration of G-CSF after bone marrow transplantation has recently been reported to promote restoration of functions injured by ischemic heart diseases and cerebral infarction [12-15]. We therefore investigated whether G-CSF increases the engraftment of transplanted bone marrow-derived cells into the olfactory epithelium damaged by methimazole [16,17] and what the most optimal timepoint was to administer G-CSF to ensure the highest engraftment rate.

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MATERIALS AND METHODS

Animals and animal care

Female GFP transgenic mice (C57BL/6 TgN (act-EGFP)OsbC14-Y01-FM131) and female C57BL/6 mice were purchased from Okayama University Animal Center. All animals used in the present study were housed, supervised, and handled according to the Okayama University Graduate School of Medicine and Dentistry Guidelines for the Care and Use of Laboratory Animals. This research was approved by the Animal Experiment Control Committee of the Okayama University Graduate School of Medicine and Dentistry, under No. OKU-2007089.

Bone marrow transplantation

Bone marrow transplantation was carried out according to a standard protocol described previously [18]. Briefly, immediately after eight-week-old female C57BL/6 recipient mice had undergone 10 Gy of lethal, whole-body-irradiation split into two doses separated by 6.5 h to minimize gastrointestinal toxicity, they were transplanted with bone marrow cells harvested from the femurs of age- and sexmatched GFP donors. Donor bone marrow cells were resuspended in Hank's balanced salt solution and $5x10^6$ bone marrow cells were injected into the tail vein of the recipients. The recipient mice were maintained in a specific pathogen-free environment and received normal chow and hyper-chlorinated drinking water for the first 3 weeks after bone marrow transplantation.

Experimental procedure

One month after bone marrow transplantation, a dose of methimazole (50mg/kg) was peritoneally administrated to damage the olfactory epithelium of recipient mice, followed by administration of G-CSF. Each dose of G-CSF (1250 μg/kg) was subcutaneously injected twice with a 3-day interval. To decide an appropriate time window of administration of G-CSF, experimental mice were divided in three groups (n = 6 in each group) by the time of the first administration of G-CSF after administration of methimazole; day 2 (group I), day 5 (group II), day 10 (group III). Control mice (no administration of G-CFS, n = 4) and experimental mice were sacrificed under diethyl-ether anesthesia at one month after the administration of methimazole. The nose of each mouse was then removed. After overnight fixation in 4% paraformaldehyde and decalcification for 14 days in 10% ethylenediaminetetraacetic acid (EDTA), the tissue samples were embedded in paraffin and 4 μm thick, consecutive cross-sections of the coronal nasal area prepared.

Immunohistochemistry for GFP

The sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked by incubation for 30 min in 0.3% H_2O_2 in methanol to block endogenous peroxidase. The sections were then incubated in 0.1% trypsin (Difco Laboratories, Detroit, MI, USA) for 5 min at 37°C. After blocking of non-specific binding sites by incubation in 10% normal goat serum containing 1%BSA for 15 min, the sections were incubated overnight at 4°C in anti-GFP polyclonal rabbit antibody (MBL, Nagoya, Japan) at a dilution of 1:1000. The sections were then rinsed in TBS followed by treatment with goat anti-rabbit immunoglobulin (Dako, Copenhagen, Denmark) antibody conjugated-HRP for 30 min. After rinsing in TBS, the sections were stained with DAB for 15 min. Staining was visualized using a light microscope (BX51-54; Olympus).

Sections from the olfactory epithelium of the donor mice were used as positive controls, while negative control sections were processed in an identical way with the exception that the primary antibody was excluded.

Double staining for GFP and olfactory marker protein (OMP), growth association protein 43 (GAP43) or cytokeratin

Double staining for GFP and OMP, GAP43 or cytokeratin was performed in order to confirm what kind of tissue was stained with GFP. After incubation of the sections in anti-GFP rabbit polyclonal antibody (1:50) or anti-GFP goat polyclonal antibody (Abcam, Cambridge, MA, USA) (1:100), anti-OMP goat polyclonal antibody (Wako Chemicals, Richmond, VA, USA)

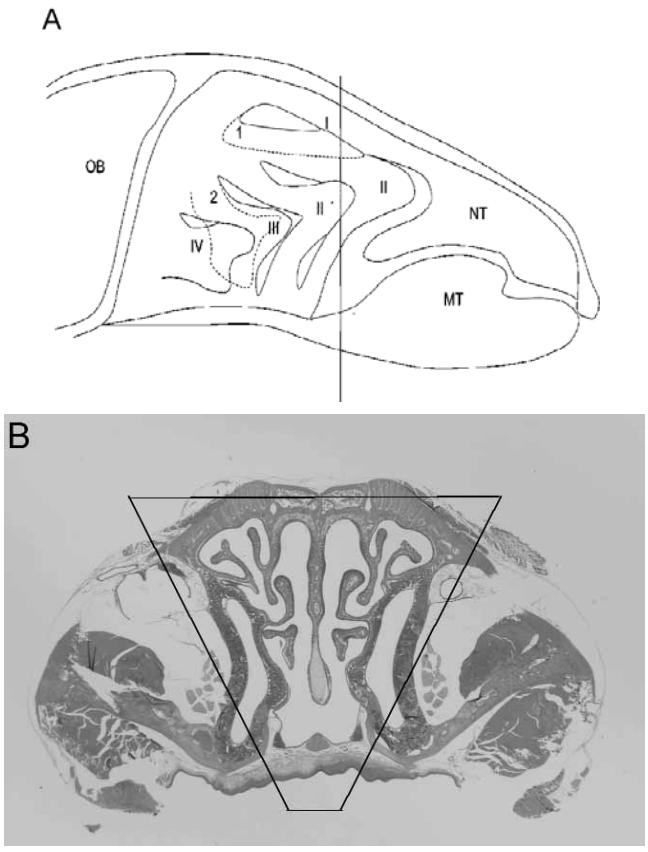

Figure 1. (A) Mouse sagittal section. NT, nasal turbinate; MT, maxillary turbinate; I IV, endoturbinates; 1 2, ectoturbinates; OB, olfactory bulb.

(B) Mouse coronal section by the line in Figure 1A. Hematoxylin–eosin staining. Positive cells were counted in the olfactory epithelium of the trapezoid of the coronal section.

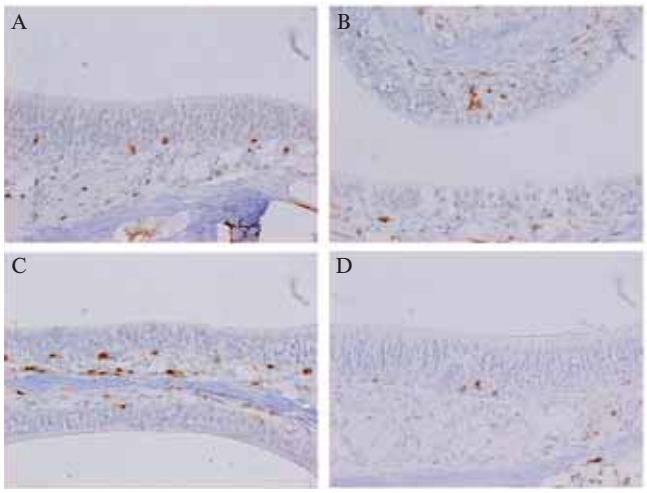

Figure 2. (A) control group, (B) group I, (C) group II, (D) group III. In each group GFP-positive cells were found not only in the olfactory epithelium but also in the submucosal layer. GFP-positive cells had spread vertically from the basal layer to the middle layer of the olfactory epithelium in the recipient mice (B). Scale bars, 20 μm

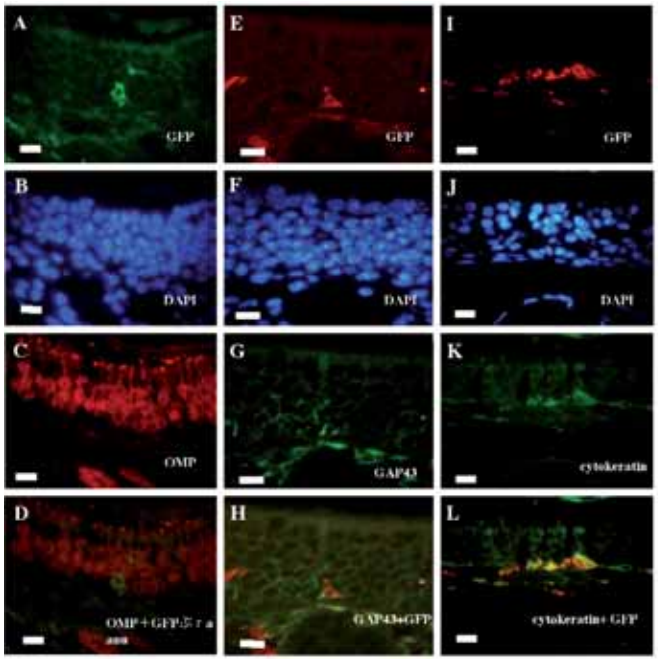

Figure 3. (A, E, I) GFP staining. (B, F, J) DAPI staining. (C) OMP staining. (G) GAP43 staining. (K) cytokeratin staining. (D) Superimposing of GFP (green) and OMP (red) shows co-localization (orange). This superimposed image shows that a portion of the donor bone marrow-derived cells became olfactory neurons. (H) Superimposing of GFP (red) and GAP43 (green) shows co-localization (orange). This superimposed image shows that a portion of the donor bone marrow- derived cells became immature olfactory neurons. (L) Superimposing of GFP (red) and cytokeratin (green) shows co-localization (orange). This superimposed image shows that a portion of the donor bone marrow-derived cells became horizontal basal cells. Scale bars, 20 μm.

(1:2000), anti-GAP43 rabbit polyclonal antibody (Chemicon, Temecula, CA, USA) (1:100) or anti-cytokeratin rabbit polyclonal antibody (Nichirei, Tokyo, Japan) (1:50) was added. Anti-rabbit chicken antibody conjugated Alexa Fluor-488 (1:250) (Invitrogen, Carlsbad, CA, USA) for GFP, cytokeratin, GAP43 and anti-goat donkey antibody conjugated with Alexa Fluor-568 (1:250) (Invitrogen) for OMP were used as the secondary antibodies.

Statistical analysis of donor bone marrow-derived cells in the olfactory epithelium

The engraftment rate of donor bone marrow-derived cells into the olfactory epithelium was calculated by counting the number of cells stained for GFP in the whole cells of the olfactory epithelium in a slice of the middle level in the coronal sections (shown in Figure 1). These slices examined and counted were representative samples of GFP staining sections. Measurements were carried out microscopically at high magnification. Data were expressed as the mean ± SD of each group. Significant differences between both groups were analyzed using one-factor analysis of variance (ANOVA) with post hoc Scheffe testing.

RESULTS

GFP-positive cells (range from 11490 to 20140 with an average value of 15020) were found in the olfactory epithelium of recipient mice in each group, including the control group (Figure 2). Double-staining for GFP and OMP, GAP43 or cytokeratin showed that a subpopulation of the GFP-positive cells in the olfactory epithelium was also stained with OMP, GAP43 or cytokeratin (Figure 3). This suggests that these cells had characteristics of olfactory mature or immature neuron cells or horizontal basal cells.

The means and standard deviations of the engraftment rate in group I, II, III and control group were 0.562 ± 0.015%, 0.505 ± 0.078, 0.315 ± 0.133, and 0.360 ± 0.055, respectively. In the groups with administration of G-CSF, the earlier G-CSF treatment after olfactory epithelium damage increased the engraftment rate of bone marrow-derived cells into the olfactory epithelium. One-factor ANOVA showed a significant difference between mean values by groups ($p = 0.0002$). The Scheffe test, a post hoc test suitable for multiple comparison, showed significant differences between group I and III ($p = 0.0008$), group I and control group ($p = 0.008$), and group II and group III ($p = 0.0088$). Any difference between the other pairs failed to reach statistical significance ($p < 0.05$) (Figure 4).

DISCUSSION

Although olfactory neurons undergo lifelong turnover under physiological and pathological conditions, an extensive loss of stem cells in the olfactory epithelium by infectious or chemical agents results in permanent anosmia. Recent advances in stem cell biology made it possible to complete some clinical studies

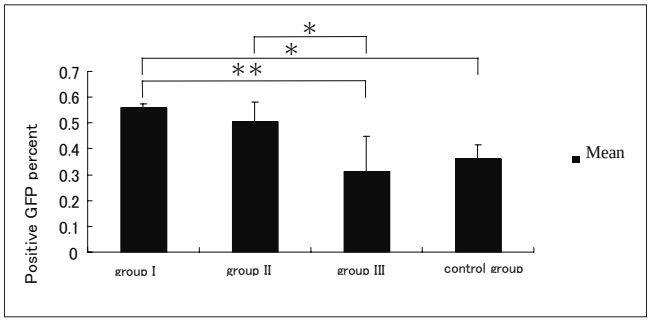

Figure 4. The percentage of GFP-positive cells to whole olfactory epithelial cells at each group. *$p < 0.01$; **$p < 0.001$ (One-factor ANOVA with post hoc Scheffe testing).

using bone marrow–derived cells for heart and vascular diseases while several are ongoing. Since meta-analysis studies of these data have demonstrated promising results [19,20], regenerative medicine using bone marrow–derived cells for olfactory disorders may provide a new strategy to resolve this intractable problem in the future.

Although we have previously reported that the engraftment rate into the olfactory epithelium by bone marrow-derived cells was 0.1%, this small engraftment rate is one of major challenges to be resolved for the clinical application of bone marrow-derived cells. In the liver the reported levels of engraftment of bone marrow-derived cells varied widely from 0.01 to 2% by types and degrees of induced injuries, cell types transplanted, and methods of detection used [3]. In the present experiment, the engraftment rate by which the administration of methimazole damaged the olfactory epithelium was 0.40%. Moreover, the engraftment rate increased up to 0.56% by adding G-CFS at the appropriate time. Although this engraftment rate is not thought to be sufficient for clinical adaptation to olfactory regeneration, the early administration of G-CSF may help to restore olfactory function.

G-CSF has the ability of mobilization stem cells from the bone marrow into the circulation and enhances the availability of circulating hematopoietic stem cells into the brain [21]. In the bone marrow hematopoietic and mesenchymal stem cells exist and there are many accumulating lines of evidence that these cells exhibit plasticity beyond their lineages [11,22], although mesenchymal stem cells are thought to have a higher plasticity than hematopoietic stem cells. Because we used non-purified bone marrow cells for transplantation, hematopoietic and mesenchymal stem cells may contribute to the differentiation into olfactory neuron cells. However, it remains controversial whether G-CSF can mobilize mesenchymal stem cells into the circulation.

Besides plasticity, the ability of bone marrow-derived cells to alter the tissue microenvironment via secretion of soluble factors may contribute more significantly than their capacity for transdifferentiation [23]. Besides mobilization, G-CSF can also induce tissue protection by inhibiting apoptosis of myocardial cells and neuron cells [12,15]. It has not been reported whether G-CSF shows directly anti-apoptotic effects to the olfactory tissue and whether the olfactory tissue has G-CSF receptors.
Kawada et al. [13] have reported that the administration of G-CSF and SCF is more effective in the subacute phase than in the acute phase of focal cerebral ischemia for enhancing cytokine-induced generation of neuronal cells from both bone marrow-derived cells and intrinsic neural stem/progenitor cells. On the other hand, our findings showed that G-CSF administration was more advantageous in the acute phase than in the subacute phase. This discrepancy may be explained from the fact that our methodology was different from theirs. Firstly, in injuries such as spinal cord contusion or focal cerebral ischemia cytokine profiles change from an initial inflammatory environment to a later tissue-regenerative environment [24]. Cell death by methimazole is caused by apoptosis, which does not induce an inflammatory process [17]. The olfactory microenvironment induced by methimazole may be favorable to tissue-regeneration from an early phase on. Secondly, olfactory neuron cells have a lifelong turnover, which is different from neurons. The regeneration of the olfactory epithelium after chemical insults is known to progress rapidly [25]. It is thought that the olfactory epithelium is regenerated up to a large degree, 10 days after methimazole administration. The above reasons might explain that the early administration of G-CSF was favorable to increase the engraftment rate of olfactory epithelium.

To maintain the homeostasis of olfactory tissue, bone marrow-derived cells need to be engrafted as stem cells of the olfactory tissue. Moreover, stemness would be more useful for repair of anosmia due to epithelial damage over the long run than addition of cells in the transit-amplifying precursor state. It is difficult to identify engrafted bone marrow-derived cells as olfactory tissue-specific stem cells because of the lack of specific markers. One possibility of certificating the engraftment of donor bone marrow-derived cells as stem cells into the olfactory epithelium, is the long existence of donor marker-positive cells in the olfactory tissue as we previously demonstrated. This indicates that donor bone marrow-derived cells may have been engrafted as stem cells [8], because of a relatively rapid turnover rate of olfactory neuron cells [26]. In the present experiment, double immunostaining showed OMP^+/GFP^+ cells, $GAP43^+/GFP^+$ cells, and $keratin^+/GFP^+$ cells. Irrespective of transdifferentiation or cell fusion, the results of double immunostaining demonstrate that donor bone marrow-derived cells most likely were engrafted as olfactory tissue-specific stem cell and differentiated into olfactory neuron cells as well as horizontal basal cells.

In conclusion, G-CSF enhanced the engraftment of bone marrow-derived cells into the olfactory epithelium. Therefore,

administration of G-CSF may contribute to the therapeutic strategy for olfactory dysfunction using bone marrow cells, although further studies need to elucidate whether G-CSF works directly on the olfactory tissue and to measure olfactory function after bone marrow transplantation following olfactory damages.

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