Original

The Ability of Transplanted Bone Marrow-Derived Cells to Differentiate into Parenchymal Cells of Salivary Glands

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Abstract: Salivary glands are important exocrine glands for oral health and initial digestion. Salivary gland dysfunction resulting from irreversible glandular damage usually leads to poor life quality in patients. Recent investigations showed that bone marrow-derived cells (BMDCs) could be grafted into non-hematopoietic cells in multiple tissues. In this study, BMDCs from green fluorescent protein (GFP) mice were transplanted into irradiated C57BL/6 mice to assess the ability of BMDCs to differentiate into parenchymal cells of salivary glands by immunohistochemistry and double fluorescent staining. The data revealed that a population of GFP positive cells showed the characteristics of acinar cells, ductal cells and myoepithelial cells in parotid and submandibular glands, and that some of BMDCs presented amylase expression. These results showed that BMDCs have the ability to differentiate into parenchymal cells of salivary glands with certain glandular cell functions. Such plasticity of BMDCs can be the first step for salivary gland regeneration by stem cells and tissue engineering therapy.

Key words: Bone marrow-derived cells, Salivary gland, Differentiation, Parenchymal cells, Green fluorescent protein

Introduction

Salivary glands are exocrine glands composed of different cells namely acinar cells, ductal cells and myoepithelial cells. Acinar cells mainly secrete protein, including amylase, proline-rich protein, etc¹⁾, and transfer water to acinar lumina chiefly by membrane water channel proteins Aquaporin 5 (AQP5), consequently producing primary saliva²⁾; ductal cells are able to modulate the composition of primary saliva³⁾; myoepithelial cells have contractile properties that aid in the secretion of saliva⁴⁾. Saliva is the main secretory product of salivary glands which plays an important role in protecting oral health and is also essential in the initial digestion of dietary starches and fats. Salivary gland dysfunction due to loss of parenchymal tissues often causes permanent discomfort in patients, which includes dryness of the

mouth, mucositis, rampant dental caries, dysphagia, etc⁵⁾. Those problems are usually managed with saliva stimulants and substitutes⁶⁾. However, the regeneration of salivary gland tissue is a more advanced procedure necessary to treat such problem. Bone marrow-derived cell (BMDC) is believed to be a source of stem cell capable of differentiating into parenchymal cells in non-hematopoietic organs like neurons in central and peripheral nervous system⁷⁻⁹⁾ and epithelium in lung¹⁰⁾, gastrointestinal tract¹¹⁾, renal¹²⁾, skin¹³⁾, etc. Furthermore, studies have shown that BMDCs could effectively ameliorate the function and stimulate tissue repair of damaged salivary glands¹⁴⁻¹⁷⁾. Lombaert et al. showed that BMDCs could be mobilized by granulocyte colony-stimulating factor (G-CSF) and homed to damaged salivary glands to improve function and morphology in mice receiving local salivary gland irradiation¹⁵⁾. Moreover, Sumita et al. found that BMDCs could rescue the functional damage of irradiated salivary glands by direct differentiation of donor BMDCs into salivary epithelial cells¹⁶⁾. Although most previous studies confirmed the success in regaining

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the function of salivary glands by BMDC therapy, few of them mentioned the type of parenchymal cell. In addition, original cell types are necessary to be renewed to regenerate salivary glands.

Therefore, to investigate the differentiation ability of BMDCs into parenchymal cells of salivary glands, BMDCs harvested from green fluorescent protein (GFP) mice were transplanted into irradiated C57BL/6 recipient mice, and their differentiation potential into component cells of salivary glands was assessed. This could be an initial step for salivary gland regeneration by stem cells and tissue engineering therapy.

Materials and Methods

Mice

Female GFP transgenic mice (C57BL/6 TgN [act-EGFP] OsbC14-Y01-FM131) were purchased from SHIMIZU Laboratory Supplies Co., Ltd. (Kyoto, Japan), and female C57BL/6 mice were purchased from Charles River Laboratories Japan Inc. (Tokyo, Japan). All experimental animals were housed in specific pathogen-free conditions 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 Care and Use Committee of Okayama University.

GFP-bone marrow chimeric experiment

The 8-week old female C57BL/6 mice were bone marrow recipients and the 8-week old female GFP transgenic mice were bone marrow donors. Bone marrow transplantation (BMT) was performed following the standard procedure previously described^{9, 18}. Briefly, bone marrow was harvested from the femurs and tibias of GFP mice and resuspended in Hank's balanced salt solution. C57BL/6 mice were exposed to a total 10 Gy of lethal whole-body-irradiation split into two doses (5 Gy, 2 times) separated by 6.5 h. The injection of 5×10^6 cells was done via tail vein into every irradiated C57BL/6 mouse immediately after irradiation. The recipient mice were fed with hyper-chlorinated drinking water and normal food during the first 3 weeks after BMT.

Tissue preparation

The recipient mice were anesthetized and sacrificed at 1 month (n=4), 2 months (n=4), 3 months (n=4) and 6 months (n=4) after bone marrow transplantation. Then, the salivary glands (parotid and submandibular glands) were harvested and fixed in 4% paraformaldehyde overnight. The samples were embedded in paraffin and serial sections of 4 μ m thickness were made.

HE staining and immunohistochemistry for GFP

Hematoxylin and eosin (HE) staining was done to visualize morphology of salivary gland tissues. GFP staining was carried out as previously described⁹. Briefly, the sections were deparaffinized, rehydrated and immersed in 0.3% H₂O₂ in methanol

for 30 min to block endogenous peroxidase. After washing with distilled water, sections were treated with 0.1% trypsin (BD Biosciences, Franklin Lakes, NJ, USA) at 37°C for 15 min. After treatment, sections were incubated with rabbit anti-GFP (MBL, Nagoya, Japan, 1:1000 dilution) polyclonal antibody at 4°C overnight. Then after, the slides were washed with tris-buffered saline (TBS) solution and then incubated with biotinylated goat anti-rabbit immunoglobulin (Nichirei, Tokyo, Japan) antibody for 30 min. After several wash with TBS, sections were incubated with streptavidin peroxidase (Nichirei) for 30 min and then stained with diaminobenzidine (DAB). Sections from salivary glands of normal female C57BL/6 mice were used as negative control.

Double-labeled fluorescent staining

Double staining was performed to identify the types and functions of GFP positive cells. AQP5, located on the cell membrane of acinar cells, was used as a marker for the acinic cell here; cytokeratin 19 (CK19), a type I intermediate filament protein and expressed in stratified and simple-type epithelia, was used here as a marker for salivary ductal cells; alpha smooth muscle actin (α -SMA) is a myoepithelial cell marker; CD31 is a transmembrane glycoprotein expressed by endothelial cells and a marker of endothelium; amylase is an important function marker of acinar cells. The primary antibodies used were the following: goat anti-GFP polyclonal antibody (abcam, Cambridge, MA, USA, 1:100 dilution), rabbit anti-GFP polyclonal antibody (MBL, 1:1000 dilution), rabbit anti-AQP5 polyclonal antibody (CALBIOCHEM, Darmstadt, Hesse-Darmstadt, Germany, 1:200 dilution), rabbit anti-CK19 polyclonal antibody (abcam, 1:100 dilution), rabbit anti- α -SMA polyclonal antibody (abcam, 1:100 dilution), rat anti-CD31 monoclonal antibody (abcam, 1:1000 dilution), goat anti-amylase polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA, 1:100 dilution). Goat anti-GFP polyclonal antibody was used for GFP with AQP5, GFP with CK19, GFP with α -SMA and GFP with CD31 double stainings, while rabbit anti-GFP polyclonal antibody was only used for GFP with amylase double staining. Tissue sections were incubated with primary antibody at 4°C overnight, washed with TBS, and then incubated for 1 h at room temperature with the following secondary antibodies: Alexa Fluor 568 donkey anti-goat IgG (Invitrogen, Carlsbad, CA, USA, 1:250 dilution), Alexa Fluor 488 chicken anti-rabbit IgG (Invitrogen, 1:250 dilution) and Alexa Fluor 488 donkey anti-rat IgG (Invitrogen, 1:250 dilution). Nuclear staining was done with DAPI.

Results

All the recipient mice were survival after irradiation and BMT. All the experimental mice were alive until they were sacrificed. The bone marrow of recipient mice was full of GFP positive cells derived from donors one month after BMT, which has been

Figure 1. (A) HE staining of salivary glands in recipient mice 1 month after BMT. (B-G) GFP positive cells in salivary glands 1 month after BMT. (B-C) Low magnification of GFP staining: numerous GFP positive cells scattered within parotid gland (B) and submandibular gland (C). (D-G) High magnification of GFP staining: (D) GFP positive acinar cells (arrow), (E) a GFP positive ductal cell (arrow), (F) GFP positive myoepithelial cells (arrows), (G) GFP positive cells around blood vessel. The bar represents 50µm.

Figure 2. Double fluorescent stainings for GFP and other makers in salivary glands after BMT. (A-C) Double staining for GFP and AQP5, 1 month after BMT. (A) GFP positive cells (stained in red) were scattered in salivary glands. (B) AQP5 was expressed on the cell membrane of acinar cells (green). (C) Co-localization of GFP, AQP5 and DAPI (blue). The picture showed an acinar cell from BMDCs (arrow). (D-F) Double staining for GFP and CK19, 6 months after BMT. (D) GFP positive cells are stained in red. (E) CK19 positive cells are stained in green. (F) Co-localization of GFP, CK19 and DAPI (blue) staining showed a BMDC became a ductal cell (arrow). (G-I) Double staining for GFP and α -SMA, 1 month after BMT. (G) GFP positive cells are stained in red; some of them were flat shape. (H) α -SMA is stained in green. (I) Co-localization of GFP, α -SMA and DAPI (blue) staining showed two GFP positive myoepithelial cells in salivary glands (arrows). (J-L) Double staining for GFP and CD31, 3 months after BMT. (J) GFP positive cells are stained in red. (K) CD31 positive cells are stained in green. (L) Co-localization of GFP, CD31 and DAPI (blue) staining showed none of endothelial cells was stained with GFP in salivary glands. The bar represents 50µm.

Figure 3. GFP and amylase double fluorescent staining of salivary glands 3 months after BMT. (A) GFP positive cells are stained in green. (B) Amylase positive cells are stained in red. (C) Co-localization of GFP, amylase and DAPI (blue) staining showed one of the cells secreting amylase was also positive to GFP in salivary glands (arrow). The bar represents 50µm.

confirmed many times in our previous studies^{9, 19}.

One month after BMT, HE staining of recipient mice revealed there was almost no apparent damage in the salivary gland parenchyma (Fig. 1A). The morphologies and structures of acini and ducts were clear. The shapes, sizes, locations and nuclei of acinic cells and ductal cells were uniform respectively. Meanwhile, GFP positive cells were found dispersedly in both of parotid gland (Fig. 1B) and submandibular gland (Fig. 1C). In both glands, distributions of GFP positive cell were observed in acinic epithelium (Fig. 1D), ductal epithelium (Fig. 1E) and myoepithelium (Fig. 1F), but no GFP positive endothelial cell was found (Fig. 1G).

Double fluorescent staining was done to identify the types and functions of cells positive to GFP. The double staining for GFP and AQP5 (Fig. 2A-C) showed that the GFP positive cell was also positive to AQP5 in salivary glands. This confirms that BMDCs can differentiate into acinar cells and are able to transfer water through such water channels. Similarly, GFP with CK19 (Fig. 2D-F) and GFP with α -SMA (Fig. 2G-I) double stainings showed that a few number of donors' BMDCs have the characteristics of ductal cells and myoepithelial cells, while we did not find double positive cell in GFP and CD31 double staining (Fig. 2J-L), suggesting that BMDCs did not differentiate into vascular endothelium in the salivary glands in our study. The GFP and amylase double staining (Fig. 3A-C) proved that the GFP positive cell contained amylase which is an important component of saliva.

Although these double positive cells (including GFP with AQP5, GFP with CK19, GFP with α -SMA and GFP with amylase double positive cells) existed, the amount of each type cells was really small. The number of typical double labeled cells was about two in each stained section of every double staining from one month to six months after BMT. Taken together, the results of double staining indicated that some of donors' BMDCs had the characters of acinic cells, ductal cells and myoepithelial cells, and excluded the possibility that all the GFP positive cells were hematopoietic cells. Moreover, some of BMDCs could produce amylase and transfer water. Altogether, the results demonstrate that BMDCs possess the capacity to differentiate into parenchymal cells of salivary glands, including acinic cells, ductal cells and myoepithelial cells in salivary glands, and furthermore some of

them have certain glandular cell functions.

Discussion

The present study tracked the engraftment of transplanted BMDCs into the parenchymal cells of salivary glands during a comparatively long experiment period and elaborated which type of parenchymal cells BMDCs could differentiate into in detail. As a result, these transplanted BMDCs quickly showed an apparent plasticity from one month post BMT in our experimental model.

The results revealed a large amount of GFP positive cells distributed in salivary glands after BMT. This coincides with previous findings, which showed that BMDCs have the possibility of migrating into many organs presenting microchimerism after transplantation in irradiated mice^{7,9,12,19-21}. Salivary gland tissues are composed of various types of cells, which originate from mesenchymal stem cells (MSCs)²². It is well known that MSCs are present in bone marrow¹⁷, so it is possible that BMDCs from donors may serve as MSCs for mesenchymal cell renewal in recipients' salivary glands. Another prediction for finding such many GFP positive cells is that salivary glands are quite sensitive to radiation²³. Therefore, BMDCs were attracted to migrate into salivary glands after BMT.

However, donor-derived GFP immunostaining may represent inflammatory cells such as lymphocytes, granulocytes, macrophages and/or other hematopoietic cells. To confirm the types of GFP positive cells and to assess the potential of BMDCs, tissue specific makers were used for double staining in this experiment. It was found that double staining of GFP and other makers were mainly in acinar cells, ductal cells and myoepithelial cells.

Nevertheless, the number of double positive cells in each section representing the BMDCs which differentiated to parenchymal cells was very few. This can be explained in two aspects: the feature of salivary glands and the experimental model. Salivary glands are well-differentiated tissues with a slow turnover and their renewal are thought to arise from either stem/progenitor dependent or progenitor independent (autologous cell division)²². Consequently, a tissue with a slow turnover may not need to replace stem cells frequently during the life span and this concurs with our previous study in olfactory epithelium⁹. A salivary gland

exposed to high dose of local head and neck radiation resulted to a comparatively high number of BMDCs differentiating into salivary epithelial cells¹⁶. In the present study, a BMT model with a certain amount of irradiation was chosen since the purpose of the experiment was to understand the ability of BMDCs to differentiate into different types of parenchymal cells.

In present study, no GFP positive cells expressed CD31 in the salivary glands of chimeric mice confirming that BMDCs did not play a role as bona fide vascular endothelium in the salivary glands. It is already known that whether BMDCs contribute to endothelial differentiation depends on their microenvironment and experimental models. It seems that BMDCs took part in the vasculature of some aggressive cancers but not in low-grade cancer, wound healing or physiologic development^{24,25}.

Although the exact mechanism of BMDC differentiation ability is unclear, it is believed that epithelial-mesenchymal interactions may have contributed in this process. Epithelial-mesenchymal interaction was initially identified in fetal development for the generation of tissues and organs^{26,27}. The morphogenesis of salivary glands depends on the interaction between oral epithelium and its mesenchyme²⁸. Epithelial-mesenchymal interaction is not only involved in embryonic development but also in tissue regeneration in adults, organ fibrosis, cancer progression, etc²⁶. Taken together, it was speculated that the same process worked in our BMT models governing the differentiation of BMDCs from the donors.

In conclusion, the study showed that BMDCs have the ability to differentiate into different types of parenchymal cell of salivary glands with certain glandular cell functions. The study can provide the basis for salivary gland regeneration by stem cells and tissue engineering therapy. Different cell populations in bone marrow possess molecular and functional heterogeneity. In order to increase the number of bone marrow derived epithelial cells, much more effort should be devoted to explore the underlying mechanism of its plasticity. Once the exact cell subpopulation has been found, the efficiency of differentiation will be greatly increased.

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