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Side Population Cells derived from Dental Pulp and Dental Germ have Distinct Surface Markers compared to Bone Marrow Side Population Cells

Takashi Okada¹, Akiko Saito², Tetsuya Amagai¹, Shoko Onodera², Yoshito Hirai¹, Masahiro Furusawa¹ and Toshifumi Azuma²

¹) Department of Endodontics and Clinical Cariology, Tokyo Dental College, Tokyo, Japan
²) Department of Biochemistry, Tokyo Dental College, Tokyo, Japan
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Abstract: The aims of the current study were to determine whether side population (SP) cell selection is possible, to show an efficient approach for purifying rat dental pulp (DP) and dental germ (DG) stem cells, and to compare phenotypic differences with bone marrow SP cells. SP cells, isolated from murine adult incisor DP and DG based on exclusion of the DNA dye Hoechst 33342, exhibited potent stem cell activity compared to main population cells. Both bone and dentin are mineralized tissues and are structurally similar. However, in contrast to bone marrow stem cells, DP stem cells do not support establishment of hematopoietic marrow. To investigate whether the phenotype of DP or DG SP cells is distinct from bone marrow SP cells, we used FACS to isolate SP cells from biologically distinct tissues (bone marrow, DP and DG) and characterized expression of several surface marker genes. Direct comparison of surface marker expression on SP cells isolated from different tissues identified surface markers common to dental tissue SP cells as well as those specific to bone marrow SP cells. FACS analysis of specific differentiation markers confirmed the similarity of surface marker expression between bone marrow SP cells and DG SP cells. However, marker expression in DP SP cells was considerably different from that in bone marrow SP cells. Because these tissues consist of multiple types of cells, heterogeneity of these tissues might explain the differences in surface marker profiles. We concluded that isolating DP SP cells with a phenotype that is distinct from that of bone marrow SP cells is possible.

Key words: CD34, CD45, FACS, Osteoblast, Stem cells

Introduction

Stem cells are generally defined as colony-derived populations that exhibit a capacity for self-renewal and differentiation into a variety of tissue lineages. Stem cells have been found in various adult tissues, including bone marrow (BM), neural tissue, muscle, and fat. Recently, stem cells were also found in adult DP cell populations. The stem cells in DP cells possess the capacity for multilineage differentiation into various cell types. Therefore, DP stem cells may represent a novel cell population with potential for future tissue engineering¹². Some pioneering studies on BM stromal cells showed that these cells are considerably heterogeneous in terms of size, morphology, enzyme histochemistry, proliferation profile, and differentiation potential⁴. Analogous results were also found in DP cells. The behavior of DP stromal cells and BM stromal cells is quite variable, even though DP stem cells are virtually identical to BM stem cells according to immunophenotyping⁵-⁷. This suggests a need for more detailed analysis. To date, few reports have been published about DP stem cells as a counterpart of BM stem cells. BM stem cells have been studied extensively and analyzed by immunophenotyping to identify stem cell fractions. A method that can isolate the stem cell fraction from the other cells present in DP has not been well developed. Side population (SP) cells, which were first isolated from murine BM via staining with the vital DNA dye Hoechst 33342, were purified and analyzed using fluorescence-activated cell sorting (FACS) and were shown to be a population of stem cells¹¹-¹³. BM SP cells are labeled faintly with Hoechst 33342 compared to the main population of cells, which exhibit brighter Hoechst 33342 staining. The faint Hoechst 33342 staining of BM SP cells was demonstrated to be due to their capacity to efflux Hoechst 33342, a process that is mediated by the ABCG2/BCRP1 transporter¹⁴. An increasing number of studies has shown isolation and characterization of SP cells from a variety of tissues including BM, adult and embryonic mouse neurospheres, embryonic stem cells, embryonic retina, adult heart, adult human pancreatic islets of Langerhans, and dental tissues¹⁵-²⁰. Although the BM-derived SP fraction comprises ~ 0.1% of the total BM, it accounts for virtually all of the hematopoietic stem...
cell (HSC) activity as demonstrated by an in vivo BM repopulation assay. An analogous population of SP cells can be purified from adult rat DP and dental germ (DG). These SP cells may contain multiple types of possible multipotential progenitors. The multipotential capacity of SP cells points to the importance of identifying molecules that are specifically expressed in SP cells. The purpose of this study was to define the surface marker profiles of SP cells isolated from two biologically distinct dental tissues, DP and DG, and to define their common features and differences. Therefore, we provide a detailed characterization of SP cells regarding the function of potential developmental pathways and further identify tissue-specific SP cell surface markers.

Materials and Methods

Animals

Five groups of six male Sprague-Dawley rats and Balb/c mice (6 weeks old) were used in this experiment. One group of six rats and mice was killed, and the jaws were dissected. The pulps were collected by manual dissection. The DG was separated at 2 mm over the tooth root. The pulps and germ tissues were pooled from each group, and duplicate analyses were performed and averaged. The experiment was approved by Tokyo Dental College Animal Experiment Committee (approval number 230401).

Hoechst and Antibody Staining for Flow Cytometry Analysis

DP and DG cells were resuspended at 10^6 cells/ml in Hank’s Balanced Salt Solution (HBSS) containing 2% FBS, 1 mM Hepes (HBSS+), and 4 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) with or without verapamil and were incubated at 37°C for 90 min. BM cells were stained with 5 μg/ml Hoechst 33342 in the same solution and incubated according to the same protocol. Cells were then stained for 30 min on ice with PE-conjugated antibodies against Sca-1 and c-kit and FITC-conjugated antibodies against CD45 and CD34 (Biosciences Pharmingen, San Diego, CA, USA). Cells were washed three times with HBSS+ and analyzed in a FACS Aria flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) followed by propidium iodide (PI) staining (2 μg/ml) to exclude dead cells. SP gates were defined using non-treated wild-type BM cells and spleen cells stained with 5 μg/ml Hoechst 33342 and 50 mM verapamil in each analysis. The Hoechst dye was excited at 350 nm, and its fluorescence was measured at two wavelengths, Hoechst blue and red, using a 405/BP30 (450/30 nm band pass filter) and a 585/BP20 (585/20 nm band pass filter) optical filter.
Figure 2. The presence of a side population (SP) of cells in dental pulp (DP) tissue and dental germ (DG) tissue in rats. The frequency of SP cells derived from DP tissue was similar to that of bone marrow SP cells, whereas the frequency of SP cells from DG tissue was higher. The mean SP rates were 0.17% in DP and 1.5% in DG.

Reverse Transcription-PCR

Total RNA was prepared from SP cell suspensions of DP and DG, and first-strand cDNA synthesis was performed using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) with an oligo-dT primer. Each first strand mixture (2 μl) was diluted in a 50 μl PCR reaction buffer: 1.5 mM MgCl₂, 200 μM each dNTP, 0.2 U EZ Taq Polymerase (Takara, Shiga, Japan), and 10 pmol of each primer set: bone sialoprotein (sense, 5'-CTATGGAGAGGACGCCACGCCTGG-3'; antisense, 5'-CATAGCCATCGTAGCCTTGTCCT-3'), osteocalcin (sense, 5'-CATGAGAGCCCTCACA-3'; antisense, 5'-AGAGCGACACCCTAGAC-3'), dentin sialophosphoprotein (DSPP) (sense, 5'-GGCAGTGACTCAAAAGGAGC-3'; antisense, 5'-TGCTGTCACTGTCACTGCTG-3'), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5'-GAGCGGACACCCCTAGAC-3'; antisense, 5'-TCATATTTGGCAGGTTTTTCT-3'). The reactions were incubated in a PCR thermal cycler at 94°C for 2 min for one cycle and then 94°C (45 s), 56°C (45 s), and 72°C (60 s) for 35 cycles. Each reaction was analyzed with 1.5% agarose gel electrophoresis, and bands were visualized with ethidium bromide staining.

Results

Isolation of SP Cells

SP cells can be isolated from aspirates of BM according to their ability to exclude Hoechst 33342 dye via the ABC transporter, ABCG2. Here, we demonstrate the presence of SP cells in DP tissue and DG tissue (Figs. 1, 2). The cells were analogous to the progeny of human BM SP cells. The frequency of SP cells derived from DP tissue was similar to BM SP cells, whereas the frequency of SP cells from DG tissue was higher compared to the incidence of BM SP cells (Figs. 1, 2). In addition, the patterns of SP frequencies of cells derived from DP tissue and DG tissue were quite similar in both rats and mice (mean 0.15% (DP) and 1.2% (DG) from mouse; mean 0.17% (DP) and 1.5% (DG) from rat).

Characterization of the Immunophenotype of SP Cells In Vitro

Although SP cells from different tissues are functionally related, they show phenotypic heterogeneity depending on the tissue of origin. We performed surface marker subtraction of SP cells and compared surface markers in SP cells obtained from different tissues. Immunohistochemical studies were performed to characterize the SP cells derived from different tissues using a...
panel of antibodies specific to known antigens. Typical immunoreactivity profiles for all cell populations are shown in Table 1. Many of the markers were not uniformly expressed, but were found in subsets of cells, indicating that the DP stem cell population is heterogeneous. Most SP cells from different tissues failed to react with the hematopoietic markers CD14 (monocyte/macrophage) and CD34 (hematopoietic stem progenitor). We found that DP SP cells and DG SP cells expressed CD45, similar to BM SP cells, but differed from those found in skin or muscle, as reported previously. The most striking difference between BM and DP was Sca-1 expression. Sca-1 was expressed in BM SP cells, but was expressed at only low levels in most DP SP cells. These differences were also seen in the expression profiles of c-kit. To our surprise, the expression of Sca-1 and c-kit was high in DG SP cells, which suggests that DG SP cells are more similar to BM SP cells than to DP SP cells.

We found that these SP cells from different tissues did not express any specific transcripts for dentin matrix components, including bone sialoprotein, osteocalcin, and DSPP, as observed with reverse transcription-PCR (Fig. 3). The bone matrix protein, bone sialoprotein, was not expressed in SP cells, whereas BM stromal cells and DP stromal cells expressed low levels of bone sialoprotein. SP cells were also negative for the odontoblast-specific marker, DSPP, which is suggestive of an undifferentiated phenotype.

Discussion

Regeneration is a remarkable feature of the human body. Cells such as blood cells are regenerated throughout life, whereas cells in most other tissues have a slower turnover. Regeneration of mineralized components in bone and dentin occurs relatively slowly, but blood cells that originate from BM stem cells are rapidly regenerated. Bone development requires a stem cell component. Although the organization of bone and dentin is quite similar and both are mineralized tissues, their clonogenic stromal cells differentiate into different cell types. BM stromal stem cells differentiate into osteoblasts, which support the establishment of hematopoietic marrow and adipocytes. On the other hand, DG tissue contains stem cells that can differentiate into osteoblasts. Nevertheless, DP stem cells and BM stem cells are virtually identical as assessed with immunophenotyping. However, each of these two stem cell populations produces quite different mineralized matrices. DP tissue does not support a hematopoietic cell population. One of the reasons why these inconsistent findings have been reported involves the difficulties with isolating the correct stem cell population. To date, HSCs have been extensively studied. Because of tissue- or developmental stage-related expression, characterization of surface marker expression after isolation of undifferentiated HSCs showed that stem cell isolation requires several flow cytometry steps. Intriguingly, a subpopulation of cells with a high Hoechst 33342 efflux ability, which were named SP cells, possesses several characteristics of stem cells. HSCs’ concentration in the SP cell population is well documented. A growing number of reports have described the existence of SP cell population in different tissues, but no reports showing SP cell population in dental tissue have been published. Here, we successfully isolated SP cell population from DP and DG from rats and mice. HSCs are well known to express Sca-1 and c-kit, whereas BM SP cells also express CD45, the common leukocyte antigen, which is not present in SP cells from different organs such as muscle. Although tissue stem cells are usually pluripotent, their surface marker profiles are different from tissue to tissue. We found that CD45 was highly expressed.
in SP cells from dental tissues. Striking differences that we found in dental tissues included expression of Sca-1 and c-kit. These differences may be related to their normal fate in these tissues. As described above, BM mesenchymal stem cells support HSCs, whereas dental mesenchymal stem cells do not. BM mesenchymal stem cells produce stromal cells called osteoblasts. Osteoblasts do not exist in DP, whereas DG is reported to contain osteoblasts\(^{35,36}\). We found that DP SP cells are quite different, especially regarding the expression of Sca-1 and c-kit. Expression of these markers in DG was similar to expression in BM. We believe that the specific environment surrounding stem cells such as SP cells is an important factor that determines these characteristics. The environment surrounding the stem cells is called the niche.

Osteoblasts are the only cell type in the niche that has been clearly demonstrated so far\(^{7,35}\). Osteoblasts are not found in dentin, and this likely has distinct effects on the phenotype of DP stem cells. In addition, our results showed that most SP cells were CD34 negative. Proliferative HSCs are mostly CD34 positive\(^{39}\). Previous reports have suggested that CD34-positive and CD45-negative cells can be expanded in culture dishes quite well. These populations of cells are also located in adipose tissue. Adipocytes exist in BM, and adipose tissue is thought to contain a large number of stem cells\(^{39}\). Although both stem cells in BM and DP could differentiate into adipocytes and osteoblasts in vitro, DP stem cells probably do not differentiate into those lineages in vivo. These differences might be due to differences in niche. CD34 is one of the most well-established stem cell markers. Previous reports have shown that CD34-positive cells proliferate more quickly than CD34-negative cells, and this may explain the poor proliferative ability of SP cells.

In summary, dental tissues have SP cell populations with distinct surface marker phenotypes from BM SP cells. Sca-1 and c-kit expression, which are prevalent in BM SP cells, were quite different in DP SP cells. These findings indicate that the DP stem cell niche may influence the stem cell phenotypes.

References

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