Effect of CD146 on Bone Regeneration by Transplantation of Stem Cells from Human Exfoliated Deciduous Teeth into Mouse Skull Defect

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Abstract

Stem cells from human exfoliated deciduous teeth (SHED) possess bone regeneration ability and may have therapeutic applications. CD146, a cell adhesion protein expressed by vascular endothelial cells, is involved in the osteoblastic differentiation of stem cells. However, the effect of CD146 on SHED-mediated bone regeneration in vivo remains unknown. Hence, in this study we aimed to establish efficient conditions for SHED transplantation. SHED were isolated from the pulp of an extracted deciduous tooth and cultured, and CD146-positive (CD146+) and CD146-negative (CD146−) populations were sorted. Heterogeneous populations of SHED and CD146+ and CD146− cells were transplanted into bone defects generated in the skulls of individual immunodeficient mice. Micro-computed tomography was performed immediately post-transplantation and at 4- and 8-weeks thereafter to evaluate bone regeneration. Histological and immunohistochemical assessments were also performed at 8 weeks after transplantation. Micro-computed tomography revealed bone regeneration upon transplantation with CD146+ and heterogeneous populations of SHED, particularly at 8 weeks after transplantation, with significantly higher bone regeneration observed following transplantation with CD146+ cells. Furthermore, histological and immunohistochemical assessments revealed that CD146+ cells promoted bone regeneration and angiogenesis. Therefore, transplantation of CD146+ SHED into bone defects may serve as a useful strategy for bone regeneration.

Introduction

Investigations of bone regenerative therapy using mesenchymal stem cells (MSCs) have been actively conducted in recent years. Bone regeneration was observed following transplantation of bone marrow mesenchymal stem cells (BMSCs) into the jaw defects of a beagle dog. However, bone marrow puncture, which is performed to collect BMSCs, causes pain and gait disturbance in animals. Therefore, we focused on stem cells from human exfoliated deciduous teeth (SHED) to develop a non-invasive and efficient bone regeneration treatment strategy. SHED have high proliferative capacity and, like BMSCs, can differentiate into osteoblasts. Thus, SHED may serve as a useful resource for bone regeneration.

CD146 is a cell adhesion molecule expressed in vascular endothelial cells and smooth muscle cells and is involved in angiogenesis and the osteoblastic differentiation of stem cells. CD146+ cells isolated from a heterogeneous cell population of MSCs exhibit higher bone regeneration capacity than CD146− cells. Thus, CD146 may promote the bone regeneration capacity of MSCs. However, in vivo promotion of bone regeneration by CD146 using SHED has not been reported.

Therefore, in this study, we investigated the effect of CD146 on bone regeneration in vivo and optimized the transplantation conditions for SHED.

Results

2.1. Isolation of CD146+ and CD146− cells
SHED collected from four patients were cultured, and the cells were sorted to isolate CD146+ and CD146− cells. The sorted CD146+ cells accounted for 83.5%, 74.1%, 89.1%, and 87.9% of the heterogeneous SHED population.

### 2.2. 3D evaluation of regenerated bone by μCT

At t₀, no regenerated bone was observed in the bone defects of all groups. At t₁ and t₂, shrinkage of the bone defect was observed in the control group; shrinkage of the bone defect with newly regenerated bone was observed at the center of the bone defect in the other groups (Fig. 1a).

The volume of regenerated bone in each group at t₁ was as follows: control group, 0.040 cm³; SHED group, 0.279 cm³; CD146+ group, 0.289 cm³; and CD146− group, 0.106 cm³. The volume of regenerated bone at t₂ was as follows in each group: control group, 0.053 cm³; SHED group, 0.172 cm³; CD146+ group, 0.317 cm³; and CD146− group, 0.111 cm³ (Fig. 1b).

### 2.3. Histological evaluation of regenerated bone

#### 2.3.1. H&E and MT staining

Newly formed bone was clearly observed in the CD146+ group, whereas insignificant bone formation was observed in the SHED group. Large blank areas were observed in the control and CD146– groups (Fig. 2a).

Mature bone was stained as red, whereas collagen fibers and osteoids were stained as blue in all groups. Only a small amount of mature bone was observed in the control and CD146– groups, whereas in the SHED and CD146+ groups, mature bone was widely observed (Fig. 2b). The area ratio of mature bone in each group was as follows: control group, 3.065%; SHED group, 6.654%; CD146+ group, 11.759%; and CD146− group, 6.098%. The area ratio of mature bone in the CD146+ group was significantly larger than that in the other groups (***p < 0.01, *p < 0.05) (Fig. 2c).

#### 2.3.2. Immunohistochemistry

Faint brown VEGF-A staining was observed throughout the transplant site in the control and CD146– groups. In contrast, in the SHED and CD146+ group, dark brown staining was observed around and at the center of the transplant site (Fig. 3a). The ratio of the area of the VEGF-stained region to the area of the transplant site was as follows in the different groups: control group, 1.008%; SHED group, 2.662%; CD146+ group, 6.977%; and CD146− group, 1.158%. For the ratio of the VEGF-stained area, the CD146+ group showed significantly higher values compared to the other groups (***p < 0.01, *p < 0.05). The SHED group showed significantly higher values than the control group (*p < 0.05) (Fig. 3b).

Furthermore, many CD31+ blood vessels were observed mainly in the SHED group and CD146+ group (Fig. 4a). The number of CD31+ blood vessels was as follows in the different groups: control group,
8.933; SHED group, 13.55; CD146+ group, 19.533; and CD146– group, 10.667. The CD146+ group showed significantly higher values than the control and CD146– group (*p < 0.05) (Fig. 4b).

2.3.4. Fluorescence immunohistochemistry

BMP-2 was weakly expressed in the upper part of the transplant site in the control group. In contrast, BMP-2 was expressed in the lower part of the transplant site in the SHED group. In the CD146+ group, the entire transplant site exhibited BMP-2 expression, whereas no expression was observed in the CD146– group (Fig. 4c).

Discussion

Human dental pulp stem cells and SHED are obtained non-invasively, reducing the physical burden on patients, and these cells have the same regenerative ability as BMSCs\textsuperscript{17-20}. However, transplantation of BMSCs does not result in significantly higher bone regeneration compared to autogenous bone graft\textsuperscript{21,22}. Hence, we focused on CD146 to determine cell transplantation conditions that could improve the bone regeneration ability of SHED. CD146 contributes to the bone differentiation and regeneration of MSCs derived from various tissues \textit{in vitro}, but its effect \textit{in vivo} remains unclear\textsuperscript{14,15,23,24}. In this study, we investigated the role of CD146 in SHED in promoting bone regeneration \textit{in vivo}.

We observed remarkable bone regeneration in the SHED and CD146+ groups, and the CD146+ group showed significantly higher regeneration than the SHED group. CD146+ cells isolated from MSCs have been reported to promote bone formation\textsuperscript{25-27}. This study revealed that CD146+ cells from SHED also contribute to bone regeneration.

In this study, CD146+ cells comprised approximately 74.1–89.1% of the total heterogeneous cell population in SHED. This indicates that a sufficient number of CD146+ cells can be isolated from a heterogeneous cell population of SHED. Thus, our approach may be useful for future clinical applications.

The CD146+ group also exhibited significantly higher expression of VEGF and BMP-2 compared with the other groups, along with the presence of a large number of CD31+ blood vessels (**p < 0.01, *p < 0.05). VEGF promotes macrophage recruitment, angiogenesis, and osteoblast differentiation in bone defects\textsuperscript{27,28}. CD31 is an adhesion molecule expressed in platelets and vascular endothelial cells and is a marker of angiogenesis\textsuperscript{29}. BMP-2 is a bone morphogenetic protein that induces the differentiation of MSCs into osteoblasts and promotes angiogenesis via VEGF expression\textsuperscript{30,31}. Our results indicated activation of angiogenesis in the CD146+ group. Interestingly, CD146 functions as a co-receptor for VEGFR-2 in endothelial cells to enhance VEGF signaling and angiogenesis via nuclear factor-κB\textsuperscript{32,33}. Therefore, CD146 may activate VEGF and the nuclear factor-κB signaling pathway to promote angiogenesis and bone regeneration.
Additionally, VEGF and BMP-2 interact in bone regeneration via MSCs\textsuperscript{34,35}. The association of BMP with the BMP receptor results in phosphorylation of Smad1, Smad5, Smad8 and regulates the expression of target genes such as \textit{VEGF} and \textit{Runx2}\textsuperscript{36}. In contrast, binding of VEGF to the VEGF receptor activates the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, culminating in increased \textit{BMP-2} expression\textsuperscript{34,35}. Thus, VEGF and BMP-2 interact and contribute to bone regeneration in MSCs. In this study, advanced bone regeneration was promoted in the CD146+ group through this mechanism.

However, the detailed effect of CD146+ cells in SHED on bone regeneration remains unclear. Therefore, it is necessary to determine the signal transduction pathways related to CD146+ cells in SHED, VEGF, and BMP-2. In addition, studies in humans are warranted to confirm the findings observed in the animal model for future clinical applications.

In conclusion, we demonstrated that CD146+ cells present in SHED are more useful for \textit{in vivo} bone regeneration than a heterogeneous population of SHED. Additionally, CD146 and VEGF may be intricately involved in bone regeneration by SHED, and further studies are required to determine their precise roles. Our study demonstrates the immense potential for the development and clinical application of SHED transplantation in bone regeneration therapy.

## Methods

### 4.1. Cell isolation and culture

Pulp tissue was collected from deciduous teeth extracted from patients being treated at the Department of Orthodontics, Hiroshima University Hospital. SHED were isolated and cultured using previously described methods\textsuperscript{16}. The guidelines pertaining to epidemiological research at Hiroshima University Hospital were strictly followed (Approval No. E-20-2). Informed consent was obtained from all participants.

### 4.2. Fluorescence-activated cell sorting

A heterogeneous population of the 3rd passage cells obtained from SHED was sorted to isolate CD146+ and CD146– cells using a FACS Aria II cell sorter (BD Biosciences, San Jose, CA, USA). The cells were stained with PE-conjugated Mouse Anti-Human CD146 (BD Pharmingen, San Jose, CA, USA) or PE-conjugated Mouse IgG1, \textgreek{k} Isotype control (BD Biosciences). The numbers of CD146+ and CD146– cells in SHED were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

### 4.3. SHED transplantation into a mouse model of bone defect

CD146+ cells, CD146– cells, and the heterogeneous SHED population were collected from the same patient and isolated by cell sorting. As SHED were of human origin, 6-week-old immunodeficient mice (BALB/c-nu; Japan Charles River International Laboratories, Inc., Yokohama, Japan) were used to avoid immunogenic and graft rejections. Non-fluorescent alfalfa-free solid food (D10001; AIN-76A; Research
Diet, EPS Masuzo, New Brunswick, NJ, USA) was administered for one week before initiating the experiment. The cells were seeded with an atelocollagen sponge (Mighty®, ø 5.0 × 1.5 mm; Koken, Tokyo, Japan) and transplanted into mouse skull defects (diameter, 5.0 mm) under general anesthesia in accordance with a previously described study. Anaesthesia consisted of midazolam (4 mg/kg; Sandoz), medetomidine (0.3 mg/kg; Orion Corp.) and butorphanol (5 mg/kg; Meiji Seika Pharma Co., Ltd.). The following groups were defined according to the implanted materials: (a) CD146+: CD146+ cells (10⁵ cells/atelocollagen sponge); (b) CD146−: CD146− cells (10⁵ cells/atelocollagen sponge); (c) SHED: heterogenous population of SHED (10⁵ cells/atelocollagen sponge); and (d) control: serum-free α-minimum essential medium (25 μL/atelocollagen sponge). In total, 20 mice were used (n=5 per group). Animal experiments were performed with permission from the Ethics Committee of Animal Experiments at Hiroshima University (Approval No. A20-81).

4.4. Three-dimensional (3D) evaluation of regenerated bone by micro-computed tomography (μCT)

The transplant site was imaged using a μCT scanner (Skyscan1176; Bruker, Billerica, MA, USA) immediately after atelocollagen transplantation (t₀), as well as at 4 (t₁) and 8 weeks after transplantation (t₂). The CT image resolution was 512 × 512 pixels, and the slice width was 35 μm. The transplant site was indicated in 3D data using ZedView (Lexi, Tokyo, Japan). The volume of regenerated bone was measured using Rapidform (Inus Technologies, Seoul, Korea) and FreeForm (SensAble Technologies, Wilmington, MA, USA).

4.5. Histological evaluation of regenerated bone

Eight weeks after atelocollagen transplantation, the immunodecient mice were euthanized and the parietal bone was extracted. The tissue specimens were decalcified using 14% ethylenediaminetetraacetic acid, embedded in paraffin, and sectioned (thickness, 7 μm) along the sagittal plane. Staining was performed as described by Hiraki et al. After staining, tissues were imaged and observed using a BZ-X800 fluorescence microscope (Keyence, Osaka, Japan).

4.5.1. Hematoxylin and eosin (H&E) and Masson’s trichrome (MT) staining

Tissue sections were deparaffinized and dehydrated, and then subjected to H&E staining and MT staining. MT staining was performed to detect mature bone. A section with atelocollagen (diameter, 5.0 mm) was used as the center of the transplant site. Using the BZ-II image analysis application (Keyence), the ratio of the area of mature bone to the area of the transplant site was calculated.

4.5.2. Immunohistochemistry

Immunostaining for vascular endothelial growth factor (VEGF) and CD31 was performed to examine angiogenesis using a slice from the center of the transplant site. The ratio of the area of the VEGF-A-stained region to the area of the transplant site and number of CD31+ blood vessels was calculated using the BZ-II image analysis application.
4.5.3. Fluorescence immunohistochemistry

Fluorescence immunohistochemistry was performed to detect the expression of bone morphogenetic protein-2 (BMP-2). Tissue sections were incubated with rabbit polyclonal anti-BMP-2 (1:250; Abcam, Cambridge, UK) at 4°C for 24 h. Alexa Fluor™ 594 goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) and 4′,6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries Ltd., Osaka, Japan) were added to the sections before observation.

4.6. Statistical analysis

All data are presented as the mean ± standard deviation. Significant differences among groups were analyzed using the Bonferroni method in BellCurve® for Excel (SSRI; Tokyo, Japan). Results with $p < 0.05$ and $p < 0.01$ were considered statistically significant.

Declarations

Data Availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

This study involving live animals follows the recommendations in the ARRIVE guidelines (PLoS Bio 8(6), e1000412, 2010).

Informed consent

Informed consent was obtained from all individual participants included in the study.

Author contributions

K.R. conceived the study. R.K., Y.Y., K.N., T.H. contributed to the study design. K.R., R.K., Y.Y wrote the main manuscript text. K.R., N.P. performed all the experiments and data analysis. Y.T., T.A., K.A., Y.H., H.N., K.T. gave critical comments on the draft of the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

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Figures
Regenerated bone evaluation by micro-computed tomography. No notable difference was detected between groups at t0. In the control- and CD146-group, a small reduction in the bone defect and regenerated bone was observed. In the SHED- and CD146+ group, several regenerated bones were found in the center of the bone defect (a). The SHED and CD146+ groups exhibited significantly higher regenerated bone mass compared to the control- and CD146– groups in the t0–t1 and t1–t2 periods. During the t1–t2 period, the CD146+ group showed significantly higher regenerated bone mass than the SHED group (b). (n = 5 for each group, ** p < 0.01, * p < 0.05).

Figure 2
Histological evaluation of regenerated bone. H&E staining showed calcified-like tissue in the CD146+ group but not in the control group (a). Masson's Trichrome staining revealed intensely stained sites showing mature bone in the SHED and CD146+ groups compared to in the control and CD146– groups (b). The proportion of areas stained red by Masson's Trichrome staining was significantly higher in the CD146+ group than in all other groups (c). (n = 5 for each group, ** p < 0.01, * p < 0.05). Scale bars = 500 μm.

**Figure 3**

Immunohistochemical analysis of VEGF expression. VEGF immunohistochemical staining was extensive and stronger in the SHED- and CD146+ groups than in the control and CD146– groups. In the CD146+ group, many stained sites were found around atelocollagen and in the lower central region (a). The proportion of VEGF-stained area was significantly higher in the CD146+ group than in all other groups (b). (n = 5 for each group, ** p < 0.01, * p < 0.05). Scale bars = 500 μm.
Figure 4

Analysis of CD31 and BMP-2 expression. Blood vessels stained using the anti-CD31 antibody were observed in all groups. Several large blood vessels were observed in the CD146+ group (a). The number of CD31+ blood vessels was high in the CD146+ group (b). Representative section depicting immunostaining with BMP-2 alone (upper panel), and co-staining with BMP-2 and DAPI (lower panel). A few sites were stained with BMP-2 in the control and CD146− groups. In the SHED group, only the lower center of the transplant site exhibited BMP-2 expression, whereas the entire transplant site in the CD146+ group showed BMP-2 expression (c). (n = 5 for each group, * p < 0.05). Scale bars = 500 μm.