Healing of Periodontal Tissues Following Transplantation of Cells in a Rat Orthodontic Tooth Movement Model

Bob N. Nayaka; William A. Wiltshire; Ben Ganss; Howard Tenenbaum; Christopher A.G. McCulloch; Charles Lekic

ABSTRACT

Objective: To determine the fate and differentiation of transplanted periodontal ligament (PL) precursor cells and mouse embryonic stem (ES) cells and their relative capacity to regenerate wounded periodontium.

Materials and Methods: Orthodontic tooth movement was introduced 24 hours before transplantation of PL or ES cells, and rats were euthanized either 24 hours or 72 hours after cell transplantation. The control rats received either no tooth movement and no cell transplantation or tooth movement and no cell transplantation. Differentiation of transplanted cells was assessed from mandibular periodontal histological tissue sections by immunohistochemical methods using monoclonal antibodies against PL cell differentiation markers. Data were analyzed using Student’s t-test at a significance level of P = .05.

Results: Transplantation of PL and ES cells resulted in a higher number of osteopontin, bone sialoprotein, and α-smooth muscle actin labeled transplanted cells, predominantly around the blood vessels of the periodontium in study rats compared with control rats (cell transplantation but no orthodontic tooth movement, P = .05). Combined treatments of tooth movement and cell transplantation resulted in enhanced regeneration of the periodontium as a result of tooth movement. Transplantation of PL cells induced a higher number of differentiating cells in the PL and alveolar bone than did transplantation of ES cells.

Conclusions: Orthodontic tooth movement promotes the differentiation of transplanted cells, and the differentiation occurs predominantly in the paravascular areas of the periodontium. In terms of regeneration of wounded periodontium, transplantation of PL cells produced a higher level of regeneration than ES cells, possibly because of PL cell plasticity and the capacity to undergo effective differentiation in the periodontal cellular microenvironment.

KEY WORDS: Orthodontic tooth movement; Cell transplantation; Differentiation; Bone marrow; Periodontal ligament; Embryonic stem cells

INTRODUCTION

Periodontal tissue healing is restricted as most periodontal ligament (PL) cells do not undergo renewal¹ and have limited mitotic activity.² Various therapeutic approaches have been developed to regulate and promote PL cell proliferation and differentiation (eg, application of extracellular matrices, growth factors, and cell transplants³,⁴) in order to facilitate restoration of periodontal tissue homeostasis. In spite of the encouraging results obtained after transplantation of epithelial cells,⁵–⁷ it is difficult to interpret these results and to distinguish between the recruitment and differentiation of host and transplanted cells.⁸,⁹

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One challenge of the data interpretation in these investigations was that the transplanted cells were not labeled and therefore it was impossible to compare their contributions to periodontal healing with those of the host cells. Previously, cell-tracking studies have demonstrated migratory behavior of lac-Z-positive cells transplanted into rat brain or adult rat liver. These results indicate that this labeling method could be used in the periodontium for assessing the fate and differentiation of transplanted cells.

The study of PL cells in steady-state conditions has limitations because of the relatively small number of proliferating cells, an overlap in the temporal expression of phenotypic markers of PL cell populations, and the existence of cells at different stages of differentiation. If PL cells could be synchronously stimulated to proliferate, then the temporal expression of matrix proteins could be more accurately related to the developmental stage of nascent cell populations, and the capacity of these cells to promote healing of periodontal tissues undergoing orthodontic tooth movement.

MATERIALS AND METHODS

After the initiation of orthodontic tooth movement, labeled PL cells (lac-Z positive cells) or ES cells (green fluorescent protein [GFP]–positive cells) were transplanted using the periodontal window wound model. Transgenic male C57BL/6J B26; 129, 10–12 week old, 25–35 g carrying *Escherichia coli* β-galactosidase lac-Z genes (Jackson Laboratories, Bar Harbor, Maine) were used to generate labeled PL cells. (GFP-labeled mouse ES cells were a gift from Dr Nagy’s lab, University of Toronto, Toronto, Ontario). Briefly, cells were isolated and cultured as described previously, except that the glucose concentration in the culture medium was increased from 1000 mg/L to 1500 mg/L as a high glucose concentration improves the cell growth of PL and ES cells.

Isolation and Culturing of PL Cells

Transgenic male C57BL/6J B26; 129, 10–12 week old, 25–35 g carrying *Escherichia coli* β-galactosidase lac-Z genes (Jackson Laboratories, Bar Harbor, Me) were used to generate labeled PL cells. (GFP-labeled mouse ES cells were a gift from Dr Nagy’s lab, University of Toronto, Toronto, Ontario). Briefly, cells were isolated and cultured as described previously, except that the glucose concentration in the culture medium was increased from 1000 mg/L to 1500 mg/L as a high glucose concentration improves the cell growth of PL and ES cells.

Animals

Thirty Sprague-Dawley male rats, 6–8 weeks old, weighing 225–250 g (Central Animal Breeding Facility, University of Manitoba) were used as recipients (Animal Protocol 00-056). Five animals were used per group. The control groups received one of two treatments: (1) no tooth movement and no cell transplantation or (2) tooth movement and no cell transplantation. The experimental groups were subjected to tooth movement and received either lac-Z-positive cells (Groups 3 and 5) or ES cells (Groups 4 and 6). Twenty-four hours before cell transplantation, tooth movement was initiated by placing elastic orthodontic ligature 0’ 120 (Ormco, Glendora, Calif) between the first and second mandibular molar. Rats were euthanized either 24 hours (Day 1, Groups 3 and 4) or 72 hours (Day 3, Groups 5 and 6) after cell transplantation.

Transplantation Procedure

Periodontal wounding was performed under general inhalation anesthesia using 1–3% halothane and oxygen:nitrous oxide (O2:N2O; 2:1). About 5 × 10^5 of lac Z (β-galactosidase) positive PL or GFP-positive ES cells were transplanted at the periodontal wound site under a dissecting microscope, and the tissue was closed with 4.0-gut suture. Rats were euthanized after intraperitoneal administration of pentobarbital at 115 mg/kg of body weight or by carbon dioxide (CO2) asphyxia.

Tissue Preparation

The decalcified specimens were washed in phosphate-buffered saline (PBS) and stained calorimetrically for β-galactosidase activity. After dehydrating specimens in cold ethanol, the plastic sections were infiltrated and embedded using Immunobed solutions and instructions (Polyscience, Warrington, Pa). The plastic sections were briefly washed with 100% ethanol and stained with either hematoxylin and eosin or 5% toluidine blue.

Immunohistochemistry

The plastic sections were briefly dehydrated and treated with 1% casein blocking solution, and then in 0.1% normal mouse serum for 1 hour in a moist chamber at room temperature. The slides were treated with 3% hydrogen peroxide in PBS for 30 minutes. They were washed in PBS for 5 minutes each and incubated with primary antibodies (ie, for osteopontin [OPN] and bone sialoprotein [BSP] mouse monoclonal antirat antibodies [Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa, USA], dilutions of 1:500 and 1:200, respectively, were used; for α-smooth muscle actin [α-SMA] mouse antirat [Sigma Chemical Co, St Louis, Mo], a dilution of 1:100 was used) and slides were then incubated in a moist chamber at 4°C overnight.
Table 1. The Ratio of Transplanted Cells and the Total Number of Cells (Expressed as Percentage ± SD) in the Paravascular Areas of the Healing PL and AB*

<table>
<thead>
<tr>
<th>Transplanted Cells</th>
<th>Periodontium Day 1/Day 3</th>
<th>AB Day 1/Day 3</th>
<th>Paravascular Day 1/Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL</td>
<td>63.0 ± 5.0/42.1 ± 3.2*</td>
<td>41.0 ± 4.8/68.0 ± 3.8*</td>
<td>71.0 ± 5.0/61.8 ± 4.5*</td>
</tr>
<tr>
<td>ES</td>
<td>43.2 ± 2.8/31.0 ± 3.6*</td>
<td>40.1 ± 3.8/44.6 ± 3.0</td>
<td>53.2 ± 2.8/31.0 ± 4.0*</td>
</tr>
</tbody>
</table>

* AB indicates alveolar bone; PL, periodontal ligament; ES, embryonic stem.
* Significant differences in the percentage of PL transplanted cells between Day 1 and Day 3 groups for the periodontium, AB, and paravascular tissues (P = .05).
* Significant difference in the percentage of ES transplanted cells in the periodontium and paravascular tissues (P = .05).

Figure 1. Photomicrograph of Day 1 group showing absence of transplanted cells at the implant site (IS). Note that transplanted cells (arrows) have not migrated into the pulp tissue (P) but only in the wounded periodontal ligament (PL). Bar = 120 μm.

The slides were washed in PBS and incubated with either avidin-biotin complex (ABC) or ABC–alkaline phosphatase (ABC-AP) reagents (Vector Laboratories, Burlington, Ontario) for 30 minutes at room temperature and then washed in two changes of PBS. The color reaction was achieved with diaminobenzidine (DAB) for 8–15 minutes. The ABC-AP color reaction was allowed to develop for 25 minutes.

Immunohistochemical Detection of Ribo-Probe Treated Sections

Slides were incubated with 5% blocking solution in maleate buffer for 30 minutes followed by treatment with 1.25 units of alkaline phosphatase conjugated to anti-digoxigenin (DIG) Fab fragments per 1 mL of blocking solution. The slides were washed twice in Tris buffer (100 mM sodium chloride [NaCl], 50 mM magnesium chloride [MgCl₂], 0.1% Tween 20, pH 9.5) to remove unbound antibody.

The colorimetric reaction was carried out using 0.2 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.2 mM nitro blue tetrazolium salt (NBT) (Boehringer Mannheim Laval, Quebec, Canada). About 125 μL of BCIP/NBT mixture was added to 5 mL of Tris buffer (pH 9.5) for 4–5 hours at 30°C. The slides were washed in distilled water and dehydrated in 70% and 100% ethanol. They were then cleared in two changes of xylol, mounted in permount, and analyzed with an Olympus digital photomicroscope, Nikon Canada, Mississauga, Ontario, Canada).

Assessment of Immunostaining

The intensity and distribution of labeled cells were evaluated under a light microscope with an intraocular grid eyepiece and an Olympus digital microscope attached with a DP 12 digital camera (Olympus, Nikon Canada, Mississauga, Ontario). The staining intensity patterns were classified as negative or positive in the event that the staining intensity clearly demonstrated presence of intracellular expression for the immunostained protein. The number of transplanted and immunolabeled cells in various landmark sites of PL and alveolar bone (AB; coronal, middle, and apical) and in paravascular areas of the periodontium was determined under 100× oil immersion lens. The GFP-positive transplanted ES cells were calculated using a Zeiss epifluorescence microscope (Zeiss Canada, Toronto, Ontario, Canada) and the number of lac-Z-positive cells was determined by the use of Olympus digital microscope. Data were analyzed using Student’s t-test at a significance level of P = .05.

RESULTS

Table 1 shows the ratio of transplanted cells and the total number of cells (expressed as percentages ± standard deviation [SD]) in the periodontium, AB, and paravascular areas of the periodontium of rats undergoing orthodontic tooth movement. The Day 1 group had a higher number of transplanted cells in the periodontium than the Day 3 group. The Day 3 group, in contrast, had a higher percentage of transplanted cells in AB than the Day 1 group. The paravascular areas in the PL had a significantly higher number of transplanted cells. Notably, all transplanted cells entered systemic circulation in less than 24 hours after the implantation of cells (Figure 1).

Figure 2 shows the differentiated α-SMA labeled...
transplanted cells around blood vessels (paravascular areas) in the PL. The differentiated OPN and BSP labeled transplanted cells in the paravascular areas are shown in Figures 3 and 4, respectively. Transplanted cells were also found within the blood vessels of the PL as well as in the periosteal and endosteal tissues of the AB. In the Day 1 group only, a number of multinucleated osteoclasts were found at the resorption sites of AB.

Percentages of OPN, BSP, and \( \alpha \)-SMA labeled transplanted and host cells and corresponding SDs for coronal, middle, and apical segments of PL and paravascular areas in the periodontium in Day 1 and Day 3 groups undergoing tooth movement are presented in Table 2. There was a significant \( (P = .05) \) increase in the percentage of labeled cells in the paravascular areas in both Day 1 and Day 3 groups. There was also a response of host cells as a result of tooth movement and transplantation. This cellular response might be...
attributable to the recruitment and activation of PL stem cells. Rats that received only transplantation without tooth movement showed about 6% of differentiated cells. Transplantation of PL and ES cells in produced an increased percentage of OPN, BSP, and α-SMA expressing cells in the PL and the AB compared with the control rats. Time-point analysis showed that within the first 6 hours after tooth movement and cell transplantation, there was an increase in the number of BSP and α-SMA labeled cells in the PL (data not shown). As time progressed, more BSP and α-SMA labeled cells were found in the PL without appreciable changes in the number of OPN labeled cells. Analysis of distribution of labeled cells in the coronal, middle, and apical segments of PL of treated rats indicate that each segment of PL had an increase in the number of OPN, BSP, and α-SMA labeled cells compared to the control rats (Table 2). At the same time there was a significant increase in the percentage of cells expressing α-SMA compared with the BSP expression in the cervical region of the PL.

Healing of wounded PL and AB was assessed by measuring the coronal, middle, and apical segment width of PL and AB in control rats and rats undergoing transplantation. These data have shown that there is an increase in the width of the AB in rats receiving cell transplantation and orthodontic tooth movement compared to the control (Table 3).

**DISCUSSION**

PL cells play a preeminent role in the regeneration of wounded periodontium and are of central importance in determining the outcome of subsequent healing.\(^{15}\) Notably, the main finding of this study was a higher degree of regeneration of the periodontium in transplanted PL cells compared with ES cells, likely because of the plasticity, level of differentiation, and tissue microenvironment of the PL cells. These findings correlate with previous studies in which transplanted cells have been shown to differentiate to lineage-specific functional cell types.\(^{16-18}\)

Tooth movement causes disruption of tissue domains in the entire PL and the surrounding AB.\(^{13}\) Shortly after application of orthodontic force, some portions of PL start to exhibit changes in width (ie, a measure of PL homeostasis\(^{14}\)). In addition, intensive tooth movement via heavy force application may cause cell death.\(^{14}\) This study has demonstrated that the PL cells are more suitable to replace destroyed periodontal ligament cells than ES cells. The fact that there was a much greater number of transplanted than repopulating host cells in wounded periodontal tissues indicates that the healing was more due to the transplanted cellular response rather than the quiescent tissue remodeling. Similarly, studies have shown that stromal mesenchymal cells, isolated from the target tissue or organ, may in fact be effective via transplantable cells for their own regeneration.\(^{19}\)

Notably, the present tooth-movement model facilitates the study of the physiology of PL cell populations because synchronized cohorts of proliferating and differentiating cells are generated shortly after stimulation.\(^{13}\) As a result, in a tooth-movement model, transplanted cells undergo migration, proliferation, and differentiation, thus repopulating lost cell populations and restoring tissue homeostasis.\(^{20}\)

A previous study showed that after systemic migration transplanted cells home to alveolar bone marrow spaces, and then proliferate and migrate to repopulate wounded periodontal tissues.\(^{14}\) Cells in periodontal tissues include multiple cellular phenotypes\(^{3}\) and are derived from proliferating precursors located at paravascular sites in the body of the PL\(^{21,22}\) or in the endostal spaces of the alveolar bone.\(^{23}\)

Subsequently, PL fibroblasts express α-SMA\(^{24}\) as well as collagen XII,\(^{25}\) and in wounded periodontal tissues expression of these proteins is an indicator of soft-tissue regeneration. Notably, during early stages of wound healing, OPN\(^{26}\) is expressed by mineralized and nonmineralized tissue-forming PL cells (reviewed by Denhardt and Guo\(^{27}\)). Previous work has shown that the expression of BSP and osteocalcin\(^{28}\) by mineralized tissue-forming cells is not normally detected until the cells have differentiated into osteoblasts, cementoblasts, or odontoblasts. However, we found that a small number of BSP labeled cells were expressed in the early stage after tooth movement and in the proximity of blood vessels. Notably, this protein is expressed almost exclusively by differentiated mineralized tissue-forming cells, and indeed our recent data indicate that transplantation of labeled PL cells into periodontal wounds leads to the differentiation of these

**Table 3.** The PL and AB Widths in Control Rats Treated with Orthodontic Tooth Movement only and Rats Treated with Cell Transplantation and Orthodontic Tooth Movement (in Millimeters)

<table>
<thead>
<tr>
<th>Segments</th>
<th>Control Group</th>
<th>Experimental Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td>PL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronal</td>
<td>0.25</td>
<td>0.23</td>
</tr>
<tr>
<td>Middle</td>
<td>0.20*</td>
<td>0.21**</td>
</tr>
<tr>
<td>Apical</td>
<td>0.28*</td>
<td>0.27**</td>
</tr>
<tr>
<td>AB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronal</td>
<td>0.18*</td>
<td>0.20**</td>
</tr>
<tr>
<td>Middle</td>
<td>0.30*</td>
<td>0.28**</td>
</tr>
<tr>
<td>Apical</td>
<td>1.44*</td>
<td>1.48**</td>
</tr>
</tbody>
</table>

* PL indicates periodontal ligament; AB, alveolar bone.
* Significant difference (\(P = .05\)) between Day 1 control group and Day 1 experimental group.
** Significant difference (\(P = .05\)) between Day 3 control group and Day 3 experimental group.
cells into osteoblasts, which have the potential of enhancing the repair process generated by tooth movement. It is conceivable that the presence of large number of cells around the blood vessels in healing PL and AB, thus expressing late-stage markers of osteogenic cell differentiation (ie, BSP), actually demonstrate that cell differentiation occurs before cell repopulation of healing sites (ie, wounded periodontium) and that transplanted cells undergo cell-type differentiation in the paravascular areas of the periodontium.

CONCLUSIONS

- In a tooth-movement model, transplanted PL or ES cells differentiate into osteogenic and nonosteogenic cell types, and this differentiation starts at or around the paravascular areas of blood vessels in the periodontium at the time or before the cells enter the wounded tissue.

- Collectively, in tissues wounded with extensive tooth movement, transplantation of undifferentiated PL progenitor/stem cells can be used to promote the healing and the restoration of cell and tissue domains.

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REFERENCES