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Assessment of bioactive and bio-adhesive therapies to enhance stem cell attachment to root surface dentine

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Abstract

Elseed MA, Murray PE, Garcia-Godoy F, Namerow KN. Assessment of bioactive and bio-adhesive therapies to enhance stem cell attachment to root surface dentine. *International Endodontic Journal*, **42**, 576–583, 2009.

Aim To compare bioactive and bio-adhesive therapies to enhance stem cell attachment to the root dentine of human teeth.

Methodology Dentine slabs (n = 72) were cut from the lower 3 mm of the roots of extracted human permanent teeth. The root dentine slabs were untreated, or coated with bio-adhesive, or human recombinant transforming growth factor-beta1 (hrTGF-B1), or human recombinant bone morphogenic protein-2 (hrBMP-2). The dentine slabs were placed with the root surface in contact with confluent periodontal stem cell (PSC) cultures using aseptic techniques. The cells and dentine slabs were submerged in culture media for 4, 24 and 72 h. The specimens were fixed in formalin, dehydrated and processed for scanning electron microscopy (SEM).

Results SEM micrographs at ×2000 magnification revealed PSC extensive adherence to root dentine for all of the bio-adhesive and bioactive treatments. The addition of bioactive molecules did not improve PSC attachment. Few cells attached to the negative control treatments.

Conclusions Bio-adhesive and bioactive growth factors were not needed to promote PSC attachment to the root dentine of human teeth, because it already appears to have good natural properties to promote PSC attachment. This suggests PSC can be used for the clinical replantation of avulsed teeth without the need for bio-adhesive and bioactive treatments.

Keywords: bone morphogenic protein-2, periodontal stem cells, scanning electron microscopy, teeth, transforming growth factor-beta1.

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Introduction

Avulsion of a tooth describes its forcible separation or complete detachment and exarticulation from the alveolar bone as a result of trauma. Avulsion injuries are considered one of the most complicated and detrimental displacement injuries of teeth. Normal healing is characterized by complete repair of the periodontal ligament (PDL) and is characterized by no radiographic signs of resorption. In a clinical study of 110 replanted teeth, 90% of teeth replanted in <30 min showed no resorption (Andreasen & Hjørting-Hansen 1966). Some replanted teeth followed for periods of up to 40 years have demonstrated complete healing with normal periodontal ligament and cementum repair (Andreasen & Hjørting-Hansen 1966). These studies indicate that the replantation of avulsed teeth can be a very successful therapy over the long term, however many replanted teeth fail. Replacement resorption is the most detrimental of the periodontal ligament responses that occur following replantation of an avulsed tooth with long

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extra-alveolar time (Tronstad 1988). Currently, there is no regenerative treatment for this condition and the prognosis of replanted teeth is significantly related to the health of the periodontal ligament cells on the root surface of the avulsed tooth (Pohl *et al.* 2005).

Regenerative endodontics may be defined as the incorporation of a combination of stem cells, tissue engineering, biomaterials and bioactive therapies to regenerate oral tissues (Murray *et al.* 2007). A goal of regenerative dentistry or regenerative endodontics must be to help improve the success of replanting avulsed teeth. A limiting factor for developing new therapies is the lack of scientific data about the ability of stem cells, bio-adhesives or bioactive molecules to stimulate the healing of avulsed replanted teeth. The recovery of periodontal stem cells (PSC) from a cryopreserved human periodontal ligament (Seo *et al.* 2005) by a team of researchers based at the National Institute of Dental and Craniofacial Research generated some excitement about the potential of these cells to be used as part of regenerative dental therapy (Ivanovski *et al.* 2006). The PSCs possess crucial stem cell properties, such as osteogenic potential (Inanç *et al.* 2006), self-renewal and multipotency, and express the mesenchymal stem cell markers (Nagatomo *et al.* 2006). Despite the ideal properties of PSC to be used to help stimulate the healing of teeth replanted after avulsion injuries, this does not appear to have been investigated. Related studies have used periodontal ligament cells to stimulate the healing of artificial bone fenestration defects in dogs with some success (Dogan *et al.* 2002, 2003). Growth factors including enamel matrix derivative, amelogenin and platelet-derived growth factor-BB have stimulated regenerative activity in cultures of human periodontal ligament cells (Chong *et al.* 2006). Transforming growth factor-beta1 (TGF-B1) and bone morphogenic protein-2 (BMP-2) have been observed to promote periodontal ligament cells proliferation (Marcopoulou *et al.* 2003). These studies demonstrate the healing potential of including stem cells and growth factors as part of dental treatment.

The purpose of this study was to examine bioactive and bio-adhesive therapies to enhance PSC attachment to the root dentine of human teeth. The rationale was to determine if PSC, with or without adjunctive therapies, may be used for the clinical replantation of avulsed teeth with the aim of developing new regenerative techniques for replacing devitalized PDL cells with PSC after prolonged extra-oral periods following tooth avulsion.

Materials and methods

Cell culture

A stock of human periodontal stem cells (PSC) was donated under a material transfer agreement from the National Institute of Dental and Craniofacial Research (NIDCR, Bethesda, MD, USA). Mouse L-929 fibroblasts were obtained from the American Tissue Culture Collection (#CCL-1; ATCC, Manassas, VA, USA). PSC and L929's were cultured in round 10×10 mm culture dishes containing Dulbecco's minimal essential media (DMEM; Gibco, Grand Island, NY, USA) supplemented with L-arginine, vitamin C, 10% fetal calf serum, and 1% antibiotics and anti-fungicides. The cells were maintained in an incubator at 37 °C in a CO_2 atmosphere until the cells reached confluence.

Preparation of substrates

Thirty-six extracted, intact permanent human teeth, which had not been stored in antibacterial or fixative solutions and had not received root canal medicaments, were selected from a bank of teeth following IRB approval. The roots of the teeth were completely cleaned using a scalpel and hand instruments. The root ends of each of the 36 teeth were then sliced into two pieces, to provide 72 root-dentine slabs, and cut 3 mm above the root end using a diamond rotary tooth-cutting saw (Materials Science; NW Ltd, Settle, UK). The dentine slabs were sterilized prior to experimentation, using a steam autoclave at 250 °C for 15 min. Six glass slides were used as controls to compare the responses of cells to another substrate, these were also autoclaved.

The dentine slabs and glass slides were added to the confluent cell cultures according to the following protocols: Group 1 – no cells were added to the culture dishes containing dentine slabs as a negative control. Group 2 – glass slides were added to culture dishes of confluent PSC as another negative control. Group 3 – the root surface of dentine slabs were placed in contact with culture dishes of confluent Mouse L929 fibroblast cells, as a positive control. Group 4 – the root surface of dentine slabs were placed in contact with culture dishes of confluent PSC. Group 5 – yhe same as group 4, plus the addition of 50 μL of human recombinant TGF-B1 to the PSC cultures prior to the placement of the dentine slabs. Group 6 – the same as group 4, plus the addition of 50 μL of human recombinant BMP-2 to the PSC cultures prior to the

Table 1 Summary of the treatment groups to be evaluated

Group number	Treatment type	Description	*In vitro* culture period (hours)/number of specimens		
			4	24	72
1	Negative control	Dentine slabs in culture without any cells	2	2	2
2	Negative control	Glass slides added to PSC	2	2	2
3	Positive control	Dentine slabs added to L929	2	2	2
4	Periodontal stem cells	Dentine slabs added to PSC	5	5	5
5	Transforming growth factor-B1	Dentine slabs coated with TGF-B1 added to PSC	5	5	5
6	Bone morphogenic protein-2	Dentine slabs coated with BMP-2 added to PSC	5	5	5
7	Bio-adhesive	Dentine slabs coated with bio-adhesive added to PSC	5	5	5

PSC, periodontal stem cells; BMP-2, bone morphogenic protein-2; TGF-B1, transforming growth factor-B1.

placement of the dentine slabs. Group 7 – the same as group 4, but the root surfaces were coated with 50 mg of Tissue-Tak bio-adhesive. Unless otherwise stated, the reagents and products were sourced from BD Biosciences (Franklin Lakes, NJ, USA). These treatment groups are shown in Table 1. The root dentine slabs and glass slides were removed from culture at 4, 24 and 72 h, according to the protocol previously described by Al-Nazhan (2004).

Preparation of samples for scanning electron microscopy

The dentine slabs were prepared for use in the scanning electron microscope (SEM) by fixing the tooth tissues in 10% neutral-buffered formalin solution at 18 °C for 24 h. The slabs were post-fixed in osmium tetroxide (1% w/v) for 2 h before being dehydrated in a graded series of alcohol solutions. Following dehydration, the specimens were removed from the solutions and placed in hexamethyldistilazane for 5 min to undergo fixation. The tooth slices were dried on filter for 30 min at room temperature. The dried specimens were fractured longitudinally using a chisel and were then mounted onto aluminum stereoscan stubs with carbon tape (Ted Pella Inc., Redding, CA, USA). The dried mounted specimens were coated with a 20–30 nm thin metallic layer of gold/palladium in a Cressington Sputter Coater model 108Auto (Watford, UK).

Scanning electron microscopy

The samples were viewed in a Quanta 200 SEM (FEI, Hilsboro, OR, USA). SEM micrographs were obtained at ×2000 magnification using digital image analysis software. Each of the dentine slab surfaces was scanned in its entirety to obtain an overview of the general surface topography. SEM micrographs were taken of representative areas characteristic of the general surface topography of each specimen to assess the effects of the test materials on the attachment of PSCs to root dentine.

Data collection and analysis

The SEM micrographs were assessed for the presence of cells using phenotypic criteria described by Al-Nazhan (2004) and summarized in Table 2. The SEM phenotypic criteria were analysed statistically using chi-square (χ^2) tests. The numbers of attached cells were counted and the results were analysed using analysis of variance (ANOVA) statistical tests. The raw data were evaluated using STATVIEW software (SAS Inc., Gary, NC, USA) at a confidence level of 95%.

Table 2 Phenotypic analysis of periodontal stem cell attachment to dentine

Criteria	Unit of measurement
1 Periodontal stem cell attachment to dentine	The number of periodontal cells attached to dentine was counted per SEM micrograph
2 Periodontal stem cell morphology	The morphology of the periodontal cells was assessed as either (i) rounded, (ii) oval filamentous cells, or (iii) flattened spreading cells

Results

Cell attachment

No cells were observed attached to root dentine prior to experimentation (Fig. 1a). These root dentine samples served as negative controls. Very few PSCs; between 1 and 3 cells per ×2000 SEM micrograph field, attached to glass between 4 and 72 h (Fig. 1b). L929 cells were used as a positive control for cell attachment. L929 cells attached to root dentine (Fig. 1c). PSC attached to root dentine between 4 and 72 h (Fig. 1d). PSC attached to dentine with TGF-B1 (Fig. 1e) and BMP-2 in the culture media (Fig. 1f). PSC also attached to root dentine that had been coated with bio-adhesive (Fig. 1g).

The glass or dentine surface appeared to influence the numbers of PSC that attached to it (ANOVA, $P = 0.0001$). The fewest numbers of PSC attached to glass (Fig. 1b), more cells were attached to the surface of root dentine (Fig. 1a,d–g). The addition of growth factors and bio-adhesive did not appear to increase the numbers of PSC attached to root dentine (ANOVA, $P = 0.4516$) (Fig. 2). The power analysis of the ANOVA test was 0.212.

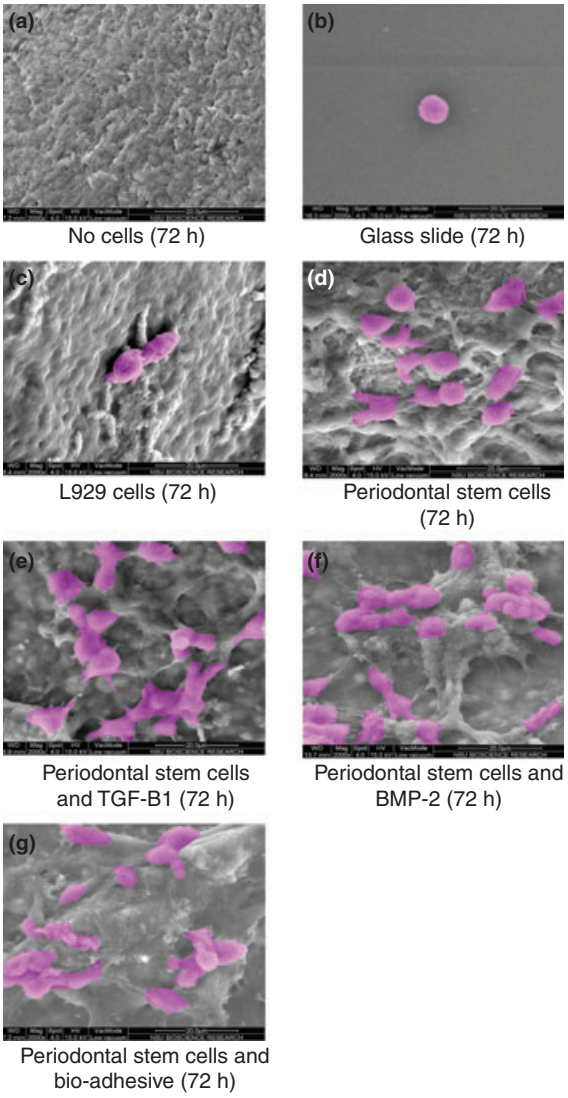

Figure 1 Phenotypes of cells attached to glass and root dentine.

Cell phenotypes

The few PSCs attached to the glass slides retained a rounded phenotype for up to 72 h, similar to the L929 cells attached to dentine (Fig. 3). The timing of PSC phenotype change when attached to dentine was similar between all the treatments (χ^2, $P = 0.4061$). The phenotype of PSCs attached to dentine gradually changed between a rounded phenotype after the first 4 h, to a flattened spreading cell phenotype after 72 h of attachment (Fig. 3). The length of time elapsed since PSC attachment to dentine was an important factor in the changes in cell phenotype that was observed (χ^2, $P = 0.0302$). The addition of BMP-2 appeared to promote a more rapid PSC phenotype change; from oval at 4 h to flattened at 24 h (Fig. 3). The same effect was not observed with TGF-B1 or the bio-adhesive (Fig. 3).

Discussion

The promising results of this pioneering investigation to study PSC attachment to root dentine may eventually provide a new approach for enhancing periodontal healing following avulsion injuries and may represent a new era of using regenerative therapies to develop definitive treatment for replacement resorption and tooth ankylosis. The translational relevance of this study is that PSC may be transplanted into the tooth socket with bio-adhesive prior to the replanting of teeth in an attempt to promote healing. Regeneration of the functional periodontium and healing without replacement resorption or tooth ankylosis would be the ultimate objective of such therapy. The authors believe that bioactive therapies to increase the success of replanted teeth must be explored, and be included as part of future regenerative endodontic treatments (reviewed by Murray *et al.* 2007).

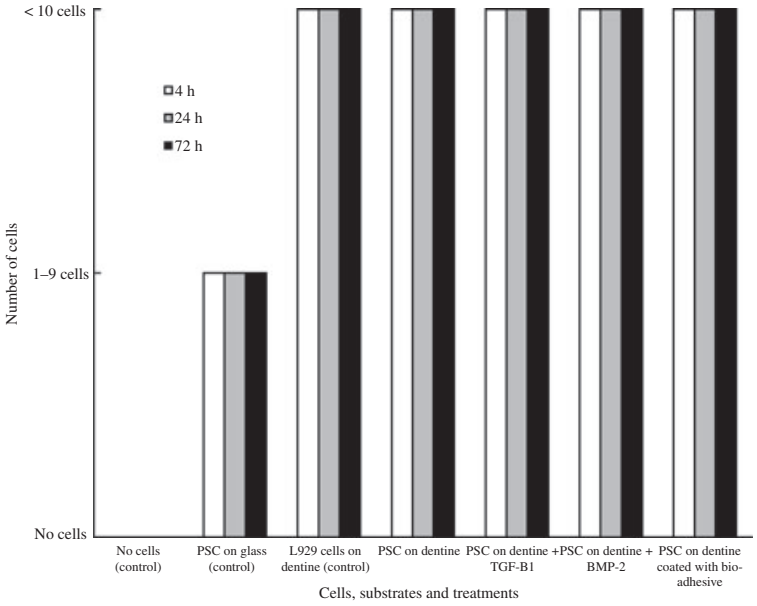

Figure 2 Bar chart of the numbers of cells attached to glass and dentine.

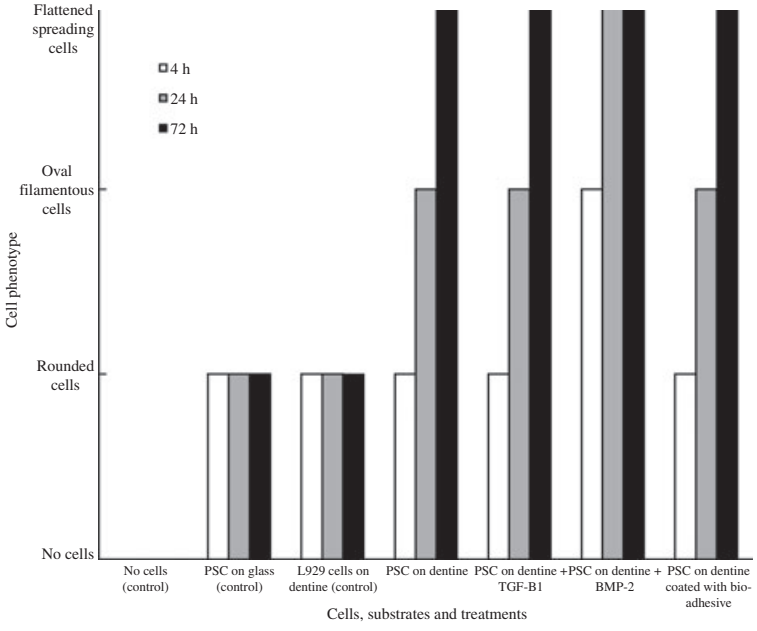

Figure 3 Bar chart of cell phenotypes in response to substrates and treatments.

If the extra-oral dry time of an avulsed tooth is more than 60 min, loss of PDL vitality may be inevitable (Tronstad 1988). Periodontal healing after replantation of avulsed teeth has been extensively published in the dental literature and several approaches have been investigated as possible means of curbing or postponing the occurrence of replacement resorption (Andreasen *et al.* 1995). Soaking of the avulsed tooth in acid prior to replantation was attempted to remove the devitalized periodontal ligament cells and hence reduce the inflammatory response after the tooth is replanted (Trope 2002). The use of 10% sodium hypochlorite to denude the root surface of devitalized periodontal ligament prior to replantation has been shown to slow down the resorptive process following replantation of monkey incisors with severely damaged periodontal membranes (Lindskog *et al.* 1985).

Preconditioning of the root surface with tetracycline, fluoride solutions and bisphosphonates to increase root resistance to resorption have been recommended by

some authors (Shulman *et al.* 1968, Björvatn *et al.* 1989, Levin *et al.* 2001). Topical application of dexamethasone has been shown to enhance periodontal healing and also to reduce the rate of progressive resorption after delayed tooth replantation in animal models (Sae-Lim *et al.* 1998, Keum *et al.* 2003). Other methods including cryopreservation and Bio-Oss together with a GTR membrane were used to assist replantation by enhancing periodontal regeneration or delaying the resorptive process (Pripatnanont *et al.* 2002, Kawasaki *et al.* 2004). Most of the above mentioned approaches were aimed at delaying the resorptive process following replantation of avulsed teeth and currently there is no definite curative or regenerative treatment for replacement resorption and ankylosis of teeth replanted after avulsion injuries.

This study presents an initial exploration of the ability of PSCs to attach to root dentine and is the first study that investigated the utilization of bioactive and bio-adhesive therapies to enhance attachment of PSC to dentine. Recent studies indicate that stem cells for cementum, dentine and the periodontal ligament also exist. Some of these cells can be expanded *in vitro*, and, embedded in a scaffold, inserted into defects to promote healing and tissue replacement (Gotlieb *et al.* 2008). In this study, SEM micrographs at ×2000 magnification showed PSC adherence to root dentine for all of the bio-adhesive and bioactive treatments. Although the addition of bioactive molecules and bio-adhesive did not improve PSC attachment, which demonstrates these bioactive procedures are not necessary to promote PSC attachment to root dentine. The power analysis of the ANOVA test was 0.212, this indicates that by greatly increasing the sample numbers, the statistical result may change, and differences in the ability of the treatments to effect PSC attachment could be observed.

No cells were observed attached to root dentine, demonstrating the teeth were completely cleaned prior to experimentation (Fig. 1a). These root dentine samples served as negative controls. The adherence of PSC to dentine was comparable to that of the positive control model represented by L929 cells. The results of the present study found that the glass or dentine surface appeared to influence the numbers of PSC that attached to it (ANOVA, $P = 0.0001$). The fewest numbers of PSC attached to glass, highlighting the importance of the surface properties for cell attachment. By comparison to glass (Fig. 1b), root dentine had a much rougher surface (Fig. 1a,d–g). The rough dentine surface appeared to promote PSC attachment, since many more attached PSC were observed (Fig. 2). In agreement with a previous study by Al-Nazhan (2004), there was a lack of PSC attachment to glass, which congruent to the previous study, establishes that cells prefer to attach to rough surfaces, rather than smooth surfaces.

Quantifying a cell phenotype provides some information about the degree of attachment a cell has with a substrate. Rounded cells have the least attachment to a surface and flattened/spreading cells have the best attachment (Sailaja *et al.* 2006). The PSC attached to the glass slides retained a rounded phenotype for up to 72 h, similar to the L929 cells attached to dentine (Fig. 3). The results show that PSC cells lacked a good attachment to the glass surface, and L929 lacked a good attachment to the dentine surface. The timing of PSC phenotype change when attached to dentine was similar between all the treatments (χ^2, $P = 0.4061$). The phenotype of PSCs attached to dentine gradually changed between a rounded phenotype after the first 4 h, to a flattened spreading cell phenotype after 72 h of attachment (Fig. 3). The length of time elapsed since PSC attachment to dentine was an important factor in the changes in cell phenotype that was observed (χ^2, $P = 0.0302$). The addition of BMP-2 appeared to promote a more rapid PSC phenotype change; from oval at 4 h to flattened at 24 h, suggesting that BMP-2 can stimulate PSC attachment to dentine (Fig. 3). The same effect was not observed with TGF-B1 or the bio-adhesive (Fig. 3), proving that these treatments lack the potential to promote PSC attachment to dentine (Fig. 3).

Among other factors, cell adhesion is considered a strategic component of wound healing. Attachment of PSC to root dentine is thought to play a critical role in periodontal tissue regeneration (Grzesik & Narayanan 2002). BMP-2 and TGF-B1 were added to PSC cultures in our study to test if they would promote PSC attachment. Both are constituents of Emdogain and are thought to be related to its effectiveness to promote periodontal regeneration (Suzuki *et al.* 2005). However, the ability of these growth factors to influence PSC attachment to dentine has not been explored previously. This *in vitro* study may serve as a first step towards developing stem cell and bioactive clinical dental therapies to enhance the healing of replanted teeth.

The issue of collecting and delineating pure cultures of human PSCs for use as part of regenerative endodontic treatment is an issue that requires much further consideration to resolve the technical and ethical dilemmas. At the present time the endodontic

profession, or potential service providers such as stem cell banks, are not trained or equipped to collect, purify, store, proliferate, and to harvest PSCs to be used as part of regenerative endodontic therapy. Much further research is needed to address the potential problems of using PSCs safely and reliably as part of regenerative endodontic treatment. Before this treatment can be routinely delivered in dental clinics, bioadhesive and stem cell-assisted replantation of avulsed teeth must be further investigated using animal models and clinical trials to investigate the interaction between PSC and avulsed teeth, optimal tooth replanting times, and to measure the healing responses *in vivo*.

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