Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 344 (2006) 362–369
BBRC
www.elsevier.com/locate/ybbrc

Novel chitosan/collagen scaffold containing transforming growth factor-β1 DNA for periodontal tissue engineering

Yufeng Zhang, Xiangrong Cheng *, Jiawei Wang, Yining Wang, Bin Shi, Cui Huang, Xuechao Yang, Tongjun Liu

Key Laboratory for Oral Biomedical Engineering Ministry of Education, School of Stomatology, Wuhan University, Wuhan 430079, PR China

Received 5 March 2006
Available online 29 March 2006

Abstract

The current rapid progression in tissue engineering and local gene delivery system has enhanced our applications to periodontal tissue engineering. In this study, porous chitosan/collagen scaffolds were prepared through a freeze-drying process, and loaded with plasmid and adenoviral vector encoding human transforming growth factor-β1 (TGF-β1). These scaffolds were evaluated in vitro by analysis of microscopic structure, porosity, and cytocompatibility. Human periodontal ligament cells (HPLCs) were seeded in this scaffold, and gene transfection could be traced by green fluorescent protein (GFP). The expression of type I and type III collagen was detected with RT-PCR, and then these scaffolds were implanted subcutaneously into athymic mice. Results indicated that the pore diameter of the gene-combined scaffolds was lower than that of pure chitosan/collagen scaffold. The scaffold containing Ad-TGF-β1 exhibited the highest proliferation rate, and the expression of type I and type III collagen up-regulated in Ad-TGF-β1 scaffold. After implanted in vivo, EGFP-transfected HPLCs not only proliferated but also recruited surrounding tissue to grow in the scaffold. This study demonstrated the potential of chitosan/collagen scaffold combined Ad-TGF-β1 as a good substrate candidate in periodontal tissue engineering.

Keywords: Chitosan; Collagen; Scaffold; Transforming growth factor-β; Gene delivery; Periodontium

Human periodontitis is an inflammatory disease affecting gingival, periodontal ligament, cementum, and alveolar bone, which could severely affect approximately 10% of the adult population and lead to bone resorption and ultimately tooth loss [1]. The final goal of periodontal therapy is to repair the damaged periodontal supporting tissues as a result of the inflammatory disease process, in which the regeneration of periodontal ligament is one of the main goals to be achieved [2]. Numerous intensive studies have been performed to improve clinical regenerative procedures in periodontal therapy [3], such as guided tissue regeneration (GTR) or an application of enamel matrix derivative [4]. Recently, researchers have focused on the regeneration of periodontal ligament (PDL) using tissue engineering [5,6]. A cell-based tissue engineering technique has been proved to be one of the most promising alternative therapies for tissue engineering [7]. This approach consists of an interactive triad of responsive cells, supportive matrix, and bioactive molecules promoting differentiation and regeneration.

A three-dimensional microenvironment is necessary in tissue engineering. Various scaffolds, such as porous sponges, mesh, fibers, and hydrogels have been prepared from biodegradable synthetic and natural polymers for the tissue engineering [8]. Chitosan is an N-deacetylation product of chitin, and it has been proved to be biologically renewable, biodegradable, biocompatible, non-antigenic, non-toxic, and bio-functional [9]. Collagen is a natural polymer that contains both acidic and basic amino acid residues. The applications of porous chitosan/collagen scaffolds for tissue engineering have been reported [10]. Tan et al. [11] reported that the addition of chitosan greatly influenced the ultrastructure of collagen and changed the collagen fiber cross-linking, which reinforced the structure and increased pore

* Corresponding author.
E-mail address: Xiangrongcheng@hotmail.com (X. Cheng).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.03.106

size. In our previous study [12], we have also found that the adherence and growth of HPLCs cultured within the chitosan/collagen scaffolds were better than those on single chitosan or collagen scaffolds. Additional, Koping et al. [13] found that chitosan was a non-toxic alternative to other cationic polymers and it could emerge as gene delivery systems.

Transforming growth factor-β (TGF-β) is a member of a large family of structurally related peptides that play a key role in regulating cell fate by controlling cell proliferation, differentiation, apoptosis, and production of extracellular matrix. In vitro the TGF-β1 has chemotactic and mitogenic activity towards gingival and periodontal ligament cells [14] and can up-regulate the production of the ECM components, including collagen, fibronectin, tenascin, and proteoglycans [15]. In vivo TGF-β1 can promote periodontal regeneration efficiently [16]. All the previous studies showed that the application of TGF-β1 could promote granulation tissue formation [14] and it may have a potential effect in promoting periodontal wound healing.

However, direct application of TGF-β1 often has limited success. A major problem that has not been overcome is how to localize the delivery of this short half-lived factor to target cells, for large doses of this factor in treatment can result in adverse side effects [17]. To address this problem and to acquire localized, continuous expression of cytokines over many days in target cells and tissue, many groups have turned to the use of gene therapy technique [18,19]. Gene therapy technique has more advantages in the local expression than continuously injecting recombinant growth factors since DNA is more stable and flexible than proteins and therefore likely to be compatible with established sustained delivery systems.

The gene-activated matrices (GAM) are a platform for gene delivery and bioreactors for seeding cells to secrete proteins [20]. Incorporation of DNA into tissue engineering matrices and its subsequent sustained release may provide an optimal means to engineer tissues. Sustained delivery of DNA from the polymer matrices may transfect large numbers of cells at localized site leading to production of a therapeutic protein to enhance tissue development.

Efficiency of gene transfer is one of the most critical factors for successful gene therapy. Numerous viral and non-viral vectors have so far been developed for this purpose, including adenovirus, retrovirus, and adeno-associated virus which are viral vectors, and cationic liposomes/polymers and HVJ liposome which are non-viral vectors [21]. Among them, recombine adenoviral vectors provide an efficient method of overexpressing cytokine genes. When introduced into periodontium, the vectors readily infect periodontal ligament cells [22] and result in production of functionally active transgene protein.

In the present study, chitosan/collagen composites combined with plasmid and virus encoding TGF-β1 gene were prepared by freeze-drying method and evaluated for cytocompatibility through seeding human periodontal ligament cells (HPLCs) into scaffold in vitro. The expression of type I and III collagen was detected with RT-PCR. Furthermore, HPLCs combined with scaffold were implanted into athymic mice to evaluate the biocompatibility in vivo.

Materials and methods

Materials. Chitosan (minimum deacetylation degree of 85%) and bovine type I collagen were obtained from Sigma (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Com. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco Company. All other reagents were of analytical grade.

The plasmid DNA was the expression vector (pEGFP, Invitrogene, USA) consisting of the coding sequence of TGF-β1 and the cytomegalovirus enhancer inserted at the upstream (pEGFP-TGF-β1). The plasmid was propagated in *Escherichia coli*, and then isolated and purified. The absorption ratio at the wavelength of 260 and 280 nm was measured for evaluation of plasmid concentration and purity.

Recombinant adenoviruses were created using the Ad-Easy system (Stratagene, La Jolla, CA). Production and purification of the recombinant virus were performed as described in detail by He et al. [23]. Briefly, the cDNA of human TGF-β1 was subcloned into pAd-Track-CMV to yield pAd-Track-TGF-β1. The resultant plasmid was contransformed into *E. coli* strain BJ5183 with the adenoviral backbone plasmid pAdEasy-1. Recombinant adenoviral plasmids were selected on kanamycin and confirmed by restriction digest. Lastly, the recombinant adenoviral plasmid was transfected into AD-293 cells where it was packaged into virus particles (Ad-TGF-β1). Viral titers were estimated by optical density and standard plaque assay. Using these methods preparations of 2×10^{10} particles/mL were obtained.

Fabrication of porous chitosan/collagen scaffolds containing DNA. Chitosan dissolved in 2% acetic acid solution was mixed with collagen powder to get 2% chitosan–collagen solution and then stirred for 2 h at 50 °C. Chitosan–collagen composites were fabricated according to the method described by Guo et al. [24]. Briefly, this mixture was put into plastic dishes at 4 °C for half an hour, and then rapidly transferred to a freezer at −35 °C overnight to solidify the solvent and induce solid–liquid phase separation. Then the solidified mixture was maintained at −80 °C for 2 h and was transferred into a freeze-drying vessel (OHRIST BETA 1-15, Germany) for 48 h until dried. The bulk porous cylinders were soaked in 0.3 M NaOH, followed by washing with double-distilled water and immersed in 75% ethanol solution for 12 h for sterilization, and then lyophilized again to get neutral, asepsis scaffolds.

Two milliliters of sterilized phosphate-buffered saline (PBS, pH 7.4) containing 1 mg pEGFP-TGF-β1 plasmid filtered through a syringe filter (pore size: 0.25 mm) for sterilization was dropped onto the dried chitosan–collagen scaffolds. The DNA content was controlled at 2 mg DNA per 1 mg scaffolds. At the same time, 1 mL adenovirus solution was dropped onto 1 mg dried chitosan–collagen scaffold, and then kept at 4 °C overnight for full incorporation of chitosan–gelatin complex with DNA and virus. These complexes were then frozen by immersion into −80 °C for 2 h and lyophilized for cell culture use.

Scaffolds were divided into three groups: group 1, the pure chitosan/collagen scaffold group; group 2, the scaffolds with pEGFP-TGF-β1 group; group 3, the scaffolds with Ad TGF-β1 group.

Human periodontal ligament cells cultured into the scaffolds. Three periodontally healthy premolars were collected from three patients who had undergone extraction for orthodontic reasons. Informed consent was obtained from all patients under a protocol approved by the Ethics Committee, School of Stomatology, Wuhan University. Human periodontal ligament cells (HPLCs) were obtained as previously described by Somerman et al. [25]. These HPLCs were used at passage 4–7 in experiments.

After 90% confluence, cells were digested by 0.25% trypsin and cell density was adjusted to 1×10^7/mL. The sterilized scaffolds were shaped into $5 \times 5 \times 1$ mm pieces and transferred into 24-well plastic culture plates. After prewetted with culture medium overnight, 100 μL cell suspensions were seeded into each scaffold. After 3 h, another 900 μL culture medium was supplied, and the culture was set at 37 °C in 5% CO_2 humidified atmosphere.

Cell–scaffold complexes were also examined with a Leica LCS Sp2 AOBS MP confocal laser scanning microscope on 3, 7, and 14 days after seeding.

The TGF-β1 secreted into culture medium was determined using a commercial TGF-β1 ELISA kit (R&D Systems Inc., Minneapolis) according to the manufacturer's instructions at day 3, 6, 9, 12, 18, and 24. Briefly, the culture medium was replaced with non-serum medium 24 h before assay, and then the supernatant was collected for evaluation. The mean values of concentration of TGF-β1 were compared by Student's t test ($n = 4$), and P values equal to or less than 0.05 were considered significant.

Scanning electron microscopy (SEM) examination. The porous morphologies of the composite scaffolds were studied by scanning electron microscopy (SEM, Hitachi X-650). The control disks were frozen in liquid nitrogen for 5 min and then cut for SEM observation. The cells were fixed on scaffold after 2 days of culture with 2.5% glutaraldehyde in 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.3) for 3 h at room temperature, rinsed three times with PBS, and dehydrated in a grade ethanol series. Samples were then critically point dried, coated with gold, and observed by SEM.

MTT assay. Scaffold was cut into $5 \times 5 \times 1$ mm pieces and transferred into 96-well plastic culture plates. A total of 10^5 HPLCs was poured onto each scaffold ($n = 6$). The cells were allowed to adhere to the scaffolds for 3 h, and then the cell–scaffold complexes were covered with 150 μL of medium. The percentage of viable cells was determined after 2, 4, and 6 days of incubation. One hundred microliters of sterile MTT (5 mg/mL) was added to each well and incubated for 3.5 h at 37 °C. At the end of incubation, the MTT solution was carefully aspirated without disturbing the pellets, and the resulting blue formazan product was solubilized in 200 μL DMSO. The optical density values were determined at least in triplicate against a reagent blank at a test wavelength of 570 nm and reference wavelength of 630 nm. The values reflect the viable cell population in each well.

Reverse transcription -polymerase chain reaction (RT-PCR). Similar to MTT, the scaffold was cut into $5 \times 5 \times 1$ mm pieces and seeded 10^6 cells in 24-well plastic culture plates. Expression of type I collagen and type III collagen was confirmed by RT-PCR, 7 and 14 days after initial seeding. The total cell RNA was prepared from each scaffold ($n = 3$) harvested at 7 and 14 days by the TaKaRa kit (TaKaRa, Japan). Total RNA (1.0 μg) was used as template for the synthesis of cDNA with OligodT and AMV reverse transcriptase (TaKaRa, Japan). The following PCR amplification reaction utilized *Taq* polymerase and specific primers. The primers for the type I collagen gene (GenBank Accession No. NM_000089) were 5′-GTG GGCTTCCTGGTGA-3′ and 5′-CTTTGGAGCCAGCTGGA-3′. The primers for the type III collagen gene(GenBank Accession No. NM_000090) were 5′-TGGCTACTTCTCGCTCTGCTT-3′ and 5′-CGG ATCCTGAGTCACAGACACA-3′. The PCR products were visualized on a 1% (w/v) agarose gel by staining with ethidium bromide and analyzed densitometrically using Gel image software. The relative levels of mRNA expression were quantified by comparison with the internal control (GAPDH). Each PCR was duplicated with the same total RNA.

In vivo study. After incubation overnight in a humidified atmosphere of 5% CO_2 in air at 37 °C, three groups of cell and scaffolds were implanted into the dorsal subcutaneous area of 6 athymic mice (BALB/c-nu; Hubei Medical Laboratory Animal Center). One group has four scaffolds. Twelve scaffolds with cells were randomly implanted into 6 mice to make sure that each athymic mouse had two scaffolds. All animal experimental procedures were carried out in accordance with the Guidelines and Regulations for the Use and Care of Animals of the Review Board of Hubei Medical Laboratory Animal Center.

The transplants were excised at 2 weeks post-transplantation, and the scaffolds were frozen-sectioned as described elsewhere [26]. Frozen sections of scaffolds and background tissue were cut at a thickness of 6 μm using a cryostat (CM1850, Leica, Germany). Serial sections were stained with hematoxylin and eosin (H&E) or used for immunohistochemistry. Primary antibodies against human TGF-β1 (Santa Cruz, CA) were used. Stained tissue was examined with a Leica DMRB fluorescence microscope.

Statistical analysis. All experiments were performed three times, with each treatment conducted in triplicate. Means and standard deviations (SD) were calculated, and the statistical significance of differences among each group was examined by one-way analysis of variance (ANOVA) and a post hoc t test. The post hoc t test was performed when the ANOVA test indicated significance at the $P < 0.05$ levels.

Results

SEM observations

As shown in Figs. 1A–C, chitosan/collagen scaffolds exhibited macroporous microstructure. The pores were

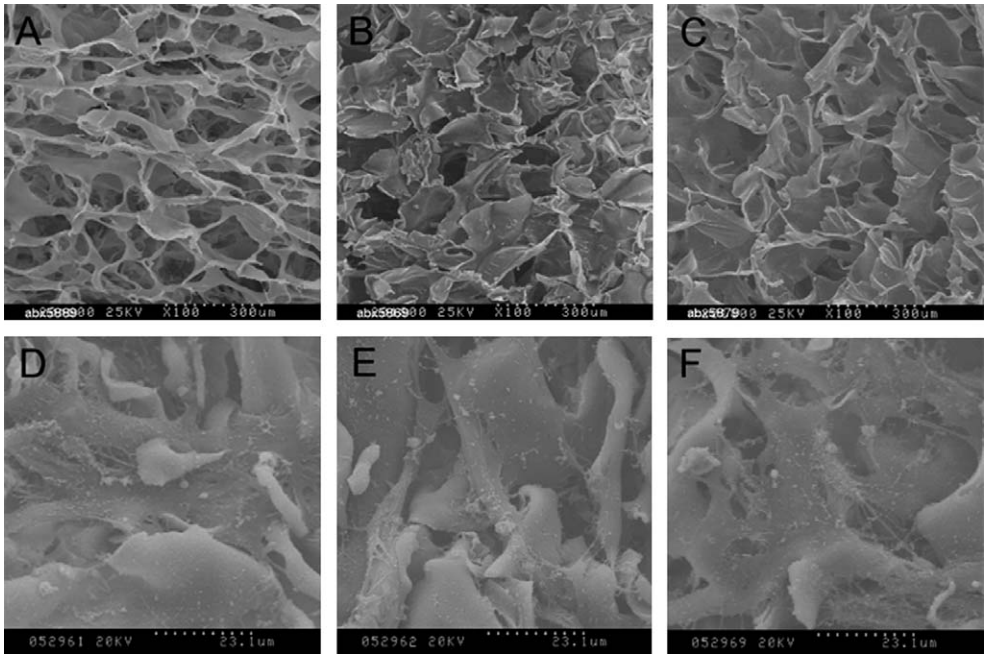

Fig. 1. SEM photographs of scaffold surface. Pure chitosan/collagen scaffold (A), the scaffolds with pEGFP-TGF-β1 (B), the scaffolds with AdTGF-β1 (C), HPLCs on pure chitosan/collagen scaffold after 2 days culture in vitro (D), HPLCs on the scaffolds with pEGFP-TGF-β1 after 2 days culture in vitro (E), and HPLCs on the scaffolds with AdTGF-β1 after 2 days culture in vitro (F).

interconnected with pore size of about 200 μm. The largest pore size could achieve 400 μm and the porosity was 80% as shown in Fig. 1A. After compounded with DNA and virus, the pore size of scaffolds decreased. Adhesion of HPLCs in the polymer scaffold was observed by SEM at day 2 after cell seeding. As shown in Figs. 1D–F, HPLCs adhered on scaffold uniformly. The cells and regenerated matrices filled in the voids. Cells adapted very closely to the surface and exhibited flattened morphology. Contact between adjacent cells was also observed in each case.

Confocal laser scanning microscope observation

To determine if these cells can be efficiently modified to express exogenous transgenes, we took confocal laser scanning microscope to observe the green fluorescent protein. The transfection efficiencies were assessed by GFP on day 3, 7, and 14. After 3 days, only a few HPLCs in group 3 appeared to be transduced by the adenovirus. After 7 days, lots of HPLCs in group 3 expressed GFP, and few HPLCs in group 2 expressed GFP (Fig. 2). This difference was statistically significant. After 14 days, the GFP expression level decreased markedly both in groups 2 and 3. Especially for group 2, the expression level decreased to nearly undetectable level.

TGF-β1 expression

The amount of secreted TGF-β1 into conditioned culture medium was determined using ELISA. HPLCs incubated in group 3 produced higher level TGF-β1 during the entire culture period. The maximum concentration of TGF-β1 in the culture media was detected after 6–9 days incubation and then followed by a moderate decline (Fig. 3). The peak concentration of the secreted proteins varied between 31 and 46 ng/ml of culture medium in human periodontal ligament cells. These results were consistent with the expression of GFP. Three weeks after the infection, human periodontal ligament cells still produced more than one-third of the maximum values.

Cell proliferation and viability

MTT assay was adopted to evaluate the cytotoxicity of the tissue engineering materials. The absorbencies of HPLCs in the scaffolds are shown in Fig. 4. It indicated

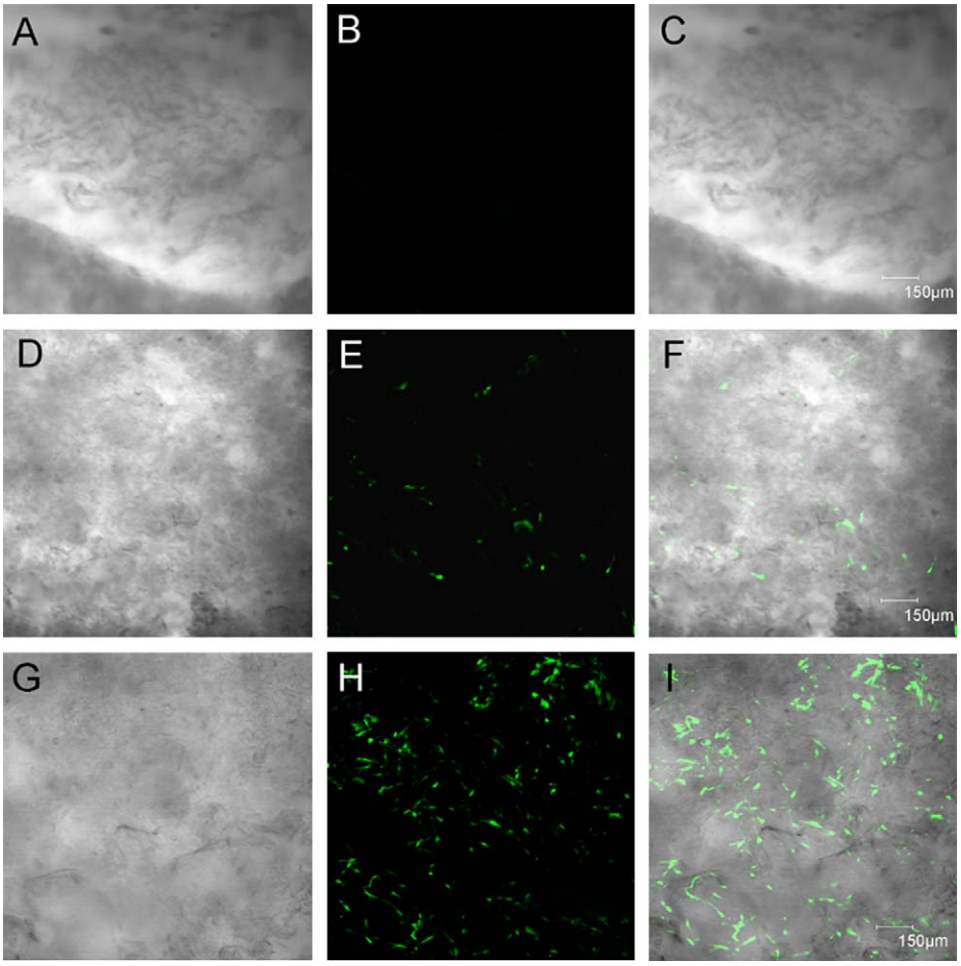

Fig. 2. Confocal LASER microscope of the scaffolds with HPLCs at the indicated times thereafter. GFP expression was visualized by fluorescence microscopy. Group 1 (A, B, and C), group 2 (D, E, and F), group 3 (G, H, and I). Confocal LASER microscopy revealed that those cells adhered, spread, and formed a monolayer in group 3.

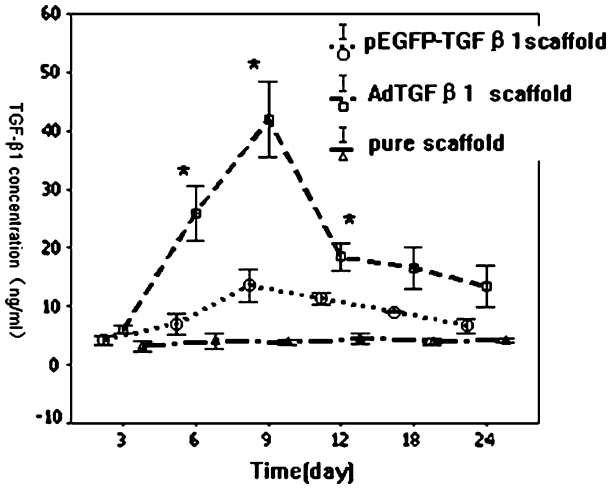

Fig. 3. The transforming growth factor-β1 expressed in the supernatants was analyzed by enzyme-linked immunosorbent assay. HPLCs incubated in group 3 produced higher level TGF-β1 during the entire culture period. The maximum concentration of TGF-β1 in the culture media was detected after 6–9 days incubation and then followed by a moderate decline, $^{*}P < 0.05$ ($n = 4$).

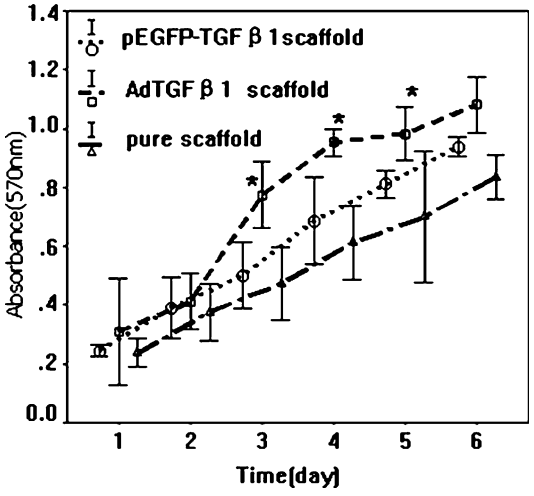

Fig. 4. The proliferation of human periodontal ligament cells in three kinds of scaffolds was measured by MTT assay. The data indicated that the percentage of viable cells on AdTGF-β1 scaffold was significantly higher than those on pEGFP-TGF-β1 scaffold and control scaffold during the culture period, $^{*}P < 0.05$ ($n = 6$).

that the percentage of viable cells on AdTGF-β1 scaffold was significantly higher than those on pEGFP-TGF-β1 scaffold and control scaffold during the culture period ($P < 0.05$), which indicated that the HPLCs showed much better proliferation properties on group 3 than on groups 1 and 2.

RT-PCR

RT-PCR was used to evaluate the influence of scaffolds on the expression of type I collagen and type III collagen. As shown in Fig. 5, the gene modified scaffold altered HPLCs genetic expression during the culture periods. The mRNA expression of type I collagen and type III collagen was up-regulated, compared to group 1. AdTGF-β1 scaffold was significantly higher than that on pEGFP-TGF-β1 scaffold ($P < 0.05$).

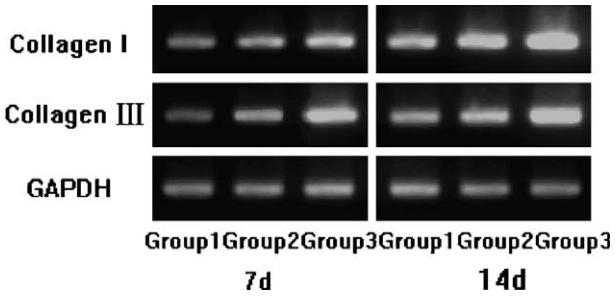

Fig. 5. RT-PCR was used to evaluate the influence of scaffolds on the expression of type I collagen and type III collagen. The gene modified scaffold altered HPLCs genetic expression during the culture periods. The mRNA expression of type I collagen and type III collagen was up-regulated, compared to group 1. AdTGF-β1 scaffold was significantly higher than that on pEGFP-TGF-β1 scaffold.

In vivo study

To investigate the biocompatibility of DNA scaffold in vivo, the athymic mouse model was adopted. HPLCs were seeded onto scaffold using a static seeding method to verify that they could adhere to these scaffolds. All twelve animals survived from the experiment. There were no inflammatory reactions, infections, or extrusions. After 2 weeks transplantation, three kinds of transplants could be clearly seen subcutaneously on the animals' dorsal area and kept the initial shape.

From frozen sections, it was found that groups 2 and 3 transplants were encapsulated by host connective tissue as shown by the absence of fluorescence in these tissues, while, human-derived cells could be readily identified in the inner side by labeled GFP under fluoromicroscope (Figs. 6B and D). Immunohistological staining with antibodies to TGF-β1 showed that only groups 2 and 3 were stained after 2 weeks transplantation. The TGF-β1 was weakly stained in group 2 and brightly stained in group 3 (Fig. 7). These immunohistological results coincided with the results of GFP expression, which suggested the AdTGF-β1 scaffold had higher transfer efficiency.

Discussion

Periodontal ligament is a connective tissue between alveolar bone and tooth cementum. Periodontal ligament cells are heterogeneous cell populations having osteoblast-like properties, including high levels of ALPase and bone-associated protein production [25]. Now periodontal ligament tissue is thought to contain pluripotent mesenchymal stem cells which can differentiate not only to connective tissue fibroblasts but also to cementum-forming cells. Successful healing, repair, or regeneration of damaged periodontal

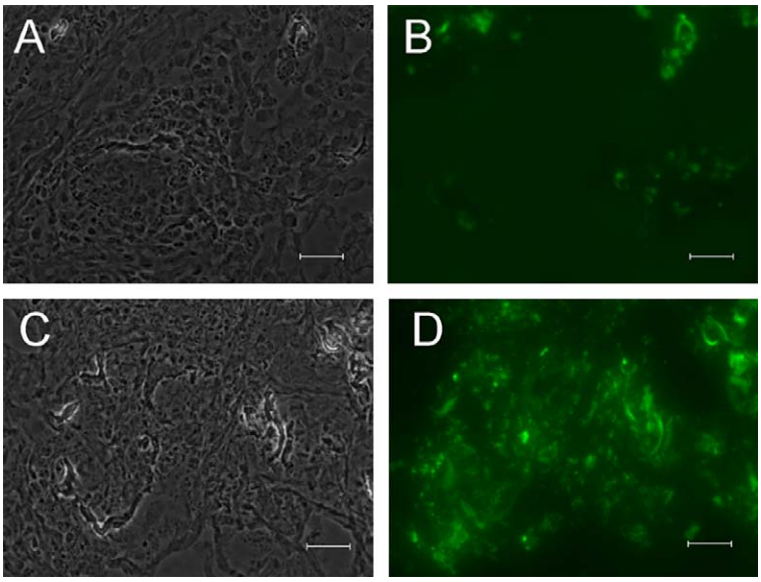

Fig. 6. A fluorescence micrograph of newly formed tissue composed of HPLCs labeled with GFP in vivo. The GFP-positive cells confirmed that the origin of newly formed tissue was the injected HPLCs (bar = 150 μm). (A) Light microscopic images of group 2; (B) fluorescence microscopic images of group 2; (C) light microscopic images of group 3; (D) fluorescence microscopic images of group 3.

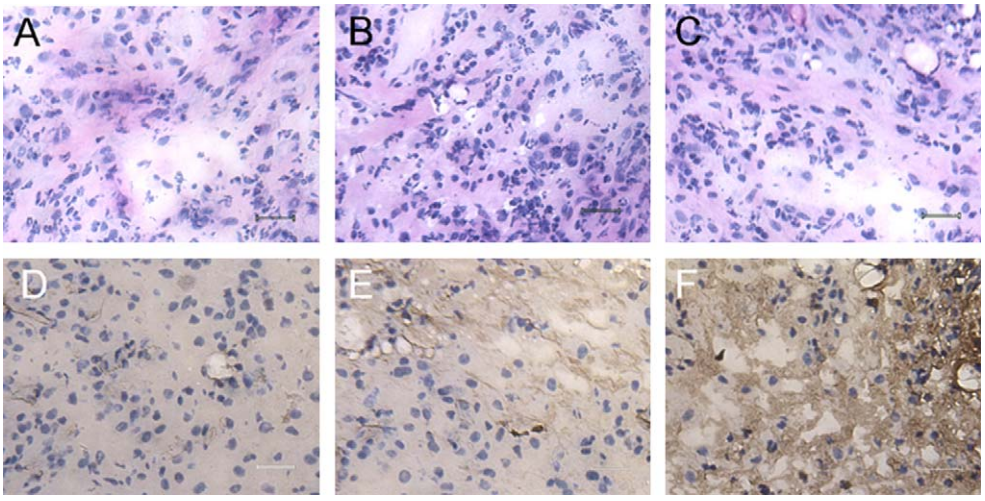

Fig. 7. Light micrographs of implants excised at 2 weeks after implantation. The cross-section was stained with hematoxylin and eosin (bar = 150 μm). (A) Pure chitosan/collagen scaffold, (B) the scaffolds with pEGFP-TGF-β1, (C) the scaffolds with AdTGF-β1. The cross-section was immunohistochemically stained with TGF-β1 antibody. (D) Group 1 was negative, (E) the TGF-β1 was weakly stained in group 2, and (F) the TGF-β1 was strongly stained in group 3.

tissue requires orchestration of such biologic events as cell migration, cell proliferation, and differentiation of pluripotent mesenchymal stem cells in the periodontal tissue [27,28]. Therefore, periodontal ligament cells are implicated in the regeneration of alveolar bone and periodontal ligament.

Cells, scaffolds, and growth factors are the three main factors for creating a tissue-engineered construct. The development of good biodegradable polymers to perform the role of a temporary matrix is an important factor in the success of cell transplantation therapy. However, these scaffolds usually do not include the bioactive molecules, including DNAs, which are required to feed the embedding cells. Therefore, the development of a biomaterial-based gene transfer method that combines gene therapy and tissue engineering to promote tissue regeneration is anticipated. The objective of this study was to evaluate two kinds of gene-combined scaffolds as prospective candidates for periodontal tissue engineering. The optimal scaffold should support the movement, proliferation, and differentiation of specific cells. In the present study, these scaffolds exhibited a well-developed pore structure and appeared to provide a good environment for the growth of HPLCs. After incubation overnight, cells adhered, spread, and formed a monolayer on the surfaces of the scaffold. HPLCs produced extracellular matrices to fill the voids of the scaffold.

The microsponges formed in the openings of chitosan/collagen scaffold provided abundant surface area for HPLCs attachment.

The aim of gene therapy is to treat diseases involving deficient or mutated proteins by delivering genes that encode intact proteins to target cells and making them express there. So the efficient delivery of growth-promoting genes locally in a sustained manner is important for effective tissue regeneration. Reporter genes have been utilized for a variety of applications ranging from gene expression and regulation to determination of efficiency of gene vector delivery. Enhanced green fluorescent protein (EGFP), a chromophore-containing protein, emits fluorescent light when excited with light at a wavelength of approximately 488 nm. Since the cells stably produced EGFP, no staining or cellular manipulation is required. This had been commonly used for reporter gene. In our study, we evaluated two different gene types combined with scaffolds for cell carriers. These two vectors could express EGFP, and we were able to visualize cells on each material by fluorescent microscopy. In addition, cell proliferation and differences in cell seeding densities on the material could be easily assessed qualitatively with fluorescent microscopy. At the same time, three dimensional cell distributions on a material could be demonstrated with the fluorescence of EGFP and confocal microscopy without the need for special preparation of the cells.

In the treatment of periodontal disease including guided tissue regeneration (GTR) therapy, it is essential that the HPLCs proliferated faster than the surrounding supporting tissue cells [4]. Therefore, the regulation of HPLCs proliferation in the periodontium is a crucial factor for periodontal tissue regeneration. Previous in vitro studies [25] have demonstrated that TGF-β1 gene-activated chitosan–gelatin matrix has a potential in the application of cartilage defect regeneration. The plasmid DNA incorporated in the scaffolds showed a burst release in the first week and a sustained release for the other 2 weeks. The gene transfected into chondrocytes expresses TGF-β1 protein stably in 3 weeks and promotes chondrocyte proliferation. In the present study, the proliferative effect of three scaffolds on HPLCs has been examined in vitro, and the scaffolds with AdTGF-β1 group obviously stimulated the cellular responses when compared with the pure chitosan/collagen scaffold and the scaffolds with pEGFP-TGF-β1 group. This may be caused by efficient expression of adenovirus vector. The proliferative effect of TGF-β1 has been reported to possess a strong effect on cell proliferation. Additionally, TGF-β1 has potential effect on periodontal regeneration for its ability to selectively inhibit epithelial cell proliferation and to stimulate periodontal ligament fibroblasts [29]. This may be a rational reason for attempts to use the scaffolds with Ad-TGF-β1 in the regeneration of periodontal tissues.

The turnover of extracellular matrix (ECM) is essential for acquisition of good repair and regeneration of PDL [30]. The main ECM component in PDL is collagen. Among which type I collagen shares approximately 80% of the total collagen in PDL [31]. With its ability to modulate collagen formation by some cells such as fibroblasts and inhibit degradation of the ECM, TGF-β1 is important in enhancement of angiogenesis and wound healing processes. Accordingly, we analyzed the effect of three kinds of scaffolds on collagen synthesis in this study, and we found that type I collagen gene level in the group 3 increased by 250% compared with group 1. This finding indicated that genetic modification of the HPLCs with TGF-β1 gene successfully elevated the ability of HPLCs to synthesize type I collagen and this genetic modification or molecular regulation with TGF-β1 gene in the HPLCs could be an efficient tool for periodontal disease therapy.

When used in vivo, however, the strong immune response resulted from the direct adenoviral gene transfer limits the duration of transgene expression and prevents the repeat treatment in the future [32]. Cell-mediated gene transfer looks like to avoid this disadvantage since in this case, cells were pre-cultured in the scaffold, and there lacked direct exposure to viral proteins. Gu et al. [33] had reported the safety, biodistribution, and immunogenicity profiles of AdPDGF-B/GAM following repeated administrations in the rabbit full-thickness dermal wound model. They investigated whether a preexisting humoral immune response to Ad would attenuate the wound healing effects of AdPDGF-B/GAM. Although AdPDGF-B DNA and PDGF-B mRNA were detected in wounds and axillary lymph nodes of treated animals, no AdPDGF-B was detected in blood or other organs. The favorable outcome of these studies supports the clinical development of GAMs for wound repair. Our results showed that immunocompetent mice could tolerate genetically modified PDLSCs that were applied into the dorsal subcutaneous area. This indirect method can also dramatically reduce the extent of vector spreading into other organs. In our study, the newly formed green fluorescence containing tissue was encapsulated by host connective tissue, which showed the absence of fluorescence.

Conclusions

The present study demonstrated the feasibility of using gene-activated chitosan/collagen scaffolds for primary HPLCs culture and expression of TGF-β1 gene in vitro. These gene-activated scaffolds showed better cytocompatibility than pure chitosan/collagen scaffold. The scaffold containing Ad-TGF-β1 exhibited the highest proliferation rate, and the expression of type I and type III collagen was up-regulated in Ad-TGF-β1 scaffold. After implanted in vivo, EGFP-transfected HPLCs not only proliferated but also recruited surrounding tissue to grow in the scaffold. This study demonstrated the potential of chitosan/collagen scaffold combined Ad-TGF-β1 as a good substrate candidate in periodontal tissue engineering.

Acknowledgment

This work was supported by National Natural Science Foundation of China (30371554).

References

[1] N.W. Schroder, D. Meister, V. Wolff, C. Christan, D. Kaner, V. Haban, P. Purucker, C. Hermann, A. Moter, U.B. Gobel, R.R. Schumann, Chronic periodontal disease is associated with single-nucleotide polymorphisms of the human TLR-4 gene, Genes Immun. 6 (2005) 448–451.

[2] H.L. Wang, T.D. Pappert, W.A. Castelli, D.J. Chiego Jr., Y. Shyr, B.A. Smith, The effect of platelet-derived growth factor on the cellular response of the periodontium: an autoradiographic study on dogs, J. Periodontal. 65 (1994) 429–436.

[3] C. Giannopoulou, G. Cimasoni, Functional characteristics of gingival and periodontal ligament fibroblasts, J. Dent. Res. 75 (1996) 895–902.

[4] A. Sculean, P. Windisch, G.C. Chiantella, N. Donos, M. Brecx, E. Reich, Treatment of intrabony defects with enamel matrix proteins and guided tissue regeneration. A prospective controlled clinical study, J. Clin. Periodontal. 28 (2001) 397–403.

[5] T. Nakahara, T. Nakamura, E. Kobayashi, K. Kuremoto, T. Matsuno, Y. Tabata, K. Eto, Y. Shimizu, In situ tissue engineering of periodontal tissues by seeding with periodontal ligament-derived cells, Tissue Eng. 10 (2004) 537–544.

[6] W.L. Murphy, D.J. Mooney, Controlled delivery of inductive proteins, plasmid DNA and cells from tissue engineering matrices, J. Periodontal. Res. 34 (1999) 413–419.

[7] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.

[8] S.J. Peter, M.J. Miller, A.W. Yasko, M.J. Yaszemski, A.G. Mikos, Polymer concepts in tissue engineering, J. Biomed. Mater. Res. 43 (1998) 422–427.

[9] S. Miyazaki, K. Ishii, T. Nadai, The use of chitin and chitosan as drug carriers, Chem. Pharm. Bull. (Tokyo) 29 (1981) 3067–3069.

[10] M.N. Taravel, A. Domard, Collagen and its interactions with chitosan, III some biological and mechanical properties, Biomaterials 17 (1996) 451–455.

[11] W. Tan, R. Krishnaraj, T.A. Desai, Evaluation of nanostructured composite collagen–chitosan matrices for tissue engineering, Tissue Eng. 7 (2001) 203–210.

[12] L. Peng, X.R. Cheng, J.W. Wang, D.X. Xu, Ge. Wang, Preparation and evaluation of porous chitosan/collagen scaffolds for periodontal tissue engineering, J. Bioact. Compat. Polym. 21 (2006) in press.

[13] M. Koping-Hoggard, I. Tubulekas, H. Guan, K. Edwards, M. Nilsson, K.M. Varum, P. Artursson, Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo, Gene Ther. 8 (2001) 1108–1121.

[14] F. Nishimura, V.P. Terranova, Comparative study of the chemotactic responses of periodontal ligament cells and gingival fibroblasts to polypeptide growth factors, J. Dent. Res. 75 (1996) 986–992.

[15] N. Matsuda, W.L. Lin, N.M. Kumar, M.I. Cho, R.J. Genco, Mitogenic, chemotactic, and synthetic responses of rat periodontal ligament fibroblastic cells to polypeptide growth factors in vitro, J. Periodontal. 63 (1992) 515–525.

[16] D.N. Tatakis, U.M. Wikesjo, S.S. Razi, T.J. Sigurdsson, M.B. Lee, T. Nguyen, B. Ongpipattanakul, R. Hardwick, Periodontal repair in dogs: effect of transforming growth factor-beta 1 on alveolar bone and cementum regeneration, J. Clin. Periodontal. 27 (2000) 698–704.

[17] B.J. Mehrara, P.B. Saadeh, D.S. Steinbrech, M. Dudziak, J.A. Spector, J.A. Greenwald, G.K. Gittes, M.T. Longaker, Adenovirus-mediated gene therapy of osteoblasts in vitro and in vivo, J. Bone Miner. Res. 14 (1999) 1290–1301.

[18] S. Wang, B.J. Baum, S. Yamano, M.H. Mankani, D. Sun, M. Jonsson, C. Davis, F.L. Graham, J. Gauldie, J.C. Atkinson, Adenoviral-mediated gene transfer to mouse salivary glands, J. Dent. Res. 79 (2000) 701–708.

[19] T.S. Rex, I. Tsui, P. Hahn, A.M. Maguire, D. Duan, J. Bennett, J.L. Dunaief, Adenovirus-mediated delivery of catalase to retinal pigment epithelial cells protects neighboring photoreceptors from photo-oxidative stress, Hum. Gene Ther. 15 (2004) 960–967.

[20] L.D. Shea, E. Smiley, J. Bonadio, D.J. Mooney, DNA delivery from polymer matrices for tissue engineering, Nat. Biotechnol. 17 (1999) 551–554.

[21] R.J. Yanez, A.C. Porter, Therapeutic gene targeting, Gene Ther. 5 (1998) 149–159.

[22] Z. Zhu, C.S. Lee, K.M. Tejeda, W.V. Giannobile, Gene transfer and expression of platelet-derived growth factors modulate periodontal cellular activity, J. Dent. Res. 80 (2001) 892–897.

[23] T.C. He, S. Zhou, L.T. da Costa, J. Yu, K.W. Kinzler, B. Vogelstein, A simplified system for generating recombinant adenoviruses, Proc. Natl. Acad. Sci. USA 95 (1998) 2509–2514.

[24] T. Guo, J. Zhao, J. Chang, Z. Ding, H. Hong, J. Chen, J. Zhang, Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-beta1 for chondrocytes proliferation, Biomaterials 27 (2006) 1095–1103.

[25] M.J. Somerman, M.F. Young, R.A. Foster, J.M. Moehring, G. Imm, J.J. Sauk, Characteristics of human periodontal ligament cells in vitro, Arch. Oral. Biol. 35 (1990) 241–247.

[26] W.G. Jiang, G. Watkins, J. Lane, G.H. Cunnick, A. Douglas-Jones, K. Mokbel, R.E. Mansel, Prognostic value of rho GTPases and rho guanine nucleotide dissociation inhibitors in human breast cancers, Clin. Cancer Res. 9 (2003) 6432–6440.

[27] T. Nakamura, M. Yamamoto, M. Tamura, Y. Izumi, Effects of growth/differentiation factor-5 on human periodontal ligament cells, J. Periodontal. Res. 38 (2003) 597–605.

[28] B.M. Seo, M. Miura, S. Gronthos, P.M. Bartold, S. Batouli, J. Brahim, M. Young, P.G. Robey, C.Y. Wang, S. Shi, Investigation of multipotent postnatal stem cells from human periodontal ligament, Lancet 364 (2004) 149–155.

[29] A.A.o.P. Committee on Research, The potential role of growth and differentiation factors in periodontal regeneration, J. Periodontal. 67 (1996) 545–553.

[30] L. Kuru, M.H. Parkar, G.S. Griffiths, H.N. Newman, I. Olsen, Flow cytometry analysis of gingival and periodontal ligament cells, J. Dent. Res. 77 (1998) 555–564.

[31] G.E. Romanos, C. Schroter-Kermani, N. Hinz, H.C. Wachtel, J.P. Bernimoulin, Immunohistochemical localization of collagenous components in healthy periodontal tissues of the rat and marmoset (*Callithrix jacchus*). I. Distribution of collagen types I and III, J. Periodontal. Res. 27 (1992) 101–110.

[32] C.H. Evans, S.C. Ghivizzani, R. Kang, T. Muzzonigro, M.C. Wasko, J.H. Herndon, P.D. Robbins, Gene therapy for rheumatic diseases, Arthritis Rheum. 42 (1999) 1–16.

[33] D.L. Gu, T. Nguyen, A.M. Gonzalez, M.A. Printz, G.F. Pierce, B.A. Sosnowski, M.L. Phillips, L.A. Chandler, Adenovirus encoding human platelet-derived growth factor-B delivered in collagen exhibits safety, biodistribution, and immunogenicity profiles favorable for clinical use, Mol. Ther. 9 (2004) 699–711.