Effect of SBD.4A – A Defined Multicomponent Preparation of Angelica sinensis – in Periodontal Regeneration Models

Hui Zhao1, Alexei Alexeev1, Vrushali Sharma1, Lorenzo Dave T. Guzman2 and Krzysztof Bojanowski1*

1Sunny BioDiscovery 2070A Walsh Ave. Santa Clara, CA 95050 and 722 East Main St. Santa Paula, CA 93060, USA
2Molecular Medicine Research Institute, 525 Del Rey Ave, Sunnyvale, CA 94085, USA

Periodontitis is a major cause of tooth motility and loss, resulting in destruction of the supporting structures of the tooth, including periodontal ligaments and alveolar bone. Periodontal surgery can slow the progression of the disease, but is costly, invasive, limited by contraindications and technique-sensitive. Recently, non-invasive pharmacological treatments using proteinaceous biologicals have become available. Here, for the first time, the bone-regenerative capabilities of a non-proteinaceous botanical – SBD.4A – a novel, stable multicomponent growth factor isolated from a medicinal plant Angelica sinensis are reported. SBD.4A was tested in osteoblast proliferation and differentiation systems, as well as in a fibroblast-secreted hyaluronic acid assay. Furthermore, SBD.4A was formulated in a slow release matrix and tested in the rat calvarial defect model. Apart from the previously reported strong stimulation of angiogenesis, fibroblast growth and collagen synthesis – the activities needed for periodontal regeneration – SBD.4A enhanced the deposition of hyaluronic acid and proliferation of osteoblasts in vitro, as well as bone regeneration in the rat calvarial defect model. Together, these results indicate the beneficial effect of SBD.4 on periodontal ligament and bone regeneration making the case for further development of this botanical growth factor. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: calvaria; periodontal; botanical; regeneration; extracellular matrix; multicomponent.

INTRODUCTION

About 70% of younger, and 90% of older, Americans suffer from periodontal disease, which often leads to irreversible alveolar bone resorption and tooth loss (Fox, 1992; Gottehrer and Berglund, 2006; Collective, 1996). Although the primary focus of periodontal therapies is to arrest disease progression, the ultimate goal should be to restore the original structure and function of the periodontium. Traditionally, surgical periodontal regeneration techniques have relied on autogenous soft tissue grafts to obtain root coverage. This therapy is being gradually complemented by guided tissue regeneration, bilaminar technique and combined periodontal regeneration techniques (Paolantonio, 2002). However, these procedures are costly, have numerous surgical contraindications, are skill-sensitive, and the recurrence of the disease sometimes requires repeated surgery. In search of alternative periodontal treatments, biological proteinaceous mediators have been tested in calvaria and alveolar bone defects in animal models (Howell et al., 1996; Takayama et al., 2001; Niikura, 2006). These factors include basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-b), vascular endothelial growth factor (VEGF) and bone morphogenetic proteins (BMP). Two proteinaceous-based treatments – GEM21S and Endogain – have been approved for clinical use. The very positive welcome and up-to-date evaluation of these products by periodontists clearly demonstrates the need for such materials. However, these proteinaceous periodontal treatments have a number of limitations, such as lability (refrigerated storage is a must) and, for Endogain, its fetal porcine source, which may raise public health and ethical concerns in some populations.

This study reports the development of an alternative therapeutic approach, using a non-proteinaceous multi-component botanical growth factor SBD.4A. SBD.4A stimulates cutaneous wound healing in healing-impaired diabetic mice and in wounds performed in human skin grafted on SCID mice (Zhao et al., 2006).

It was hypothesized that SBD.4A could be also beneficial for periodontal regeneration, because it stimulates the activities (angiogenesis, collagen deposition and fibroblast proliferation) required for periodontal lesion healing. Furthermore, Angelica sinensis components were reported to stimulate osteoblast growth, in vitro (Yang et al., 2002). Therefore SBD.4A was tested for in vitro and in vivo bone-related activities, such as osteoblast proliferation and differentiation, as well as hyaluronic acid (HA) secretion. Furthermore, in order to examine the bioactivity of SBD.4A in an in vivo model, the rat calvarial defect system, broadly employed in the evaluation of periodontium-regenerative materials, was used (for example see Develioglu et al., 2006; Pang et al., 2005; Blom et al.,

Received 22 June 2007
Revised 27 September 2007
Accepted 4 October 2007

Copyright © 2008 John Wiley & Sons, Ltd.
Together, these experiments aimed to determine whether SBD.4A has potential for, and can find applications in, periodontal bone regeneration and, by extension, in orthopedics and reconstructive surgery.

**MATERIALS AND METHODS**

**Preparation of SBD.4A.** SBD.4A was extracted from roots of *Angelica sinensis* (Gansu province, PR China). The raw material was purchased from FuDe Biological and Food Co. Ltd (Ningxia, PR China) and authenticated by Professor Pang Qi Yan from the Institute of Research on the Quality of Natural Resources, at the Ningxia University of Agriculture and Forestry, where the voucher was also deposited (specimen # PB-18).

The root was cut into ~5 mm pieces and briefly extracted in room-temperature type I water obtained through filtration of deionized water with the Barnstead (Dubuque, IA) EasyPure UF water purification system. The extract was further filtered through a Nalgene (Rochester, NY) 0.22 μm pore filter, followed by filtration through a 3 kD cut off centrifugal filter device (Millipore, Bedford, MA). The molecular characterization, HPLC and LC-MS profiles of SBD.4A have been published elsewhere (Zhao et al., 2006).

**In vitro cell-based assays**

**Cells and cell culture reagents.** Cell culture reagents were from HyClone, (Logan, UT), except alpha medium (Gibco, Grand Island, NY). MC3T3 osteoblasts (ATCC, Manassas, VA) were grown in 10% alpha medium/90% DMEM/1% fetal bovine serum (FBS). PC-3 prostate and MDA MB231 breast cancer cells (ATCC) were cultured in RPMI 1640/2.5% FBS, human neonatal dermal fibroblasts (Cambrex, Walkersville, MD) were grown in DMEM/2.5% FBS, and human breast epithelial adenocarcinoma cells MDA-MB-231 (ATCC) and bovine endothelial capillary cells (BCE, Cambrex) were grown in DMEM (1 g/L glucose)/5% calf serum (CS). All cell-based studies were performed in 96-well tissue culture plates (Falcon brand) and the volume of test materials added was 5% of the final volume. At the end of all studies, cell numbers were determined by colorimetric quantification of sulforhodamine B (Sigma, St Louis, MO) staining at 575 nm in 3550-UV microplate reader (BioRad, Hercules, CA), as described before (Skehan et al., 1990).

**Cell proliferation assay.** Cells were seeded at 2500 cells/well, SBD.4A and controls were added and cell culture was continued for 3 days. Cell numbers were determined as described above.

**Osteoblast differentiation.** MC3T3 osteoblasts were seeded at 10 000 cells/well, SBD.4A and controls were added and the culture pursued for 3 days. The marker for osteoblast differentiation – alkaline phosphatase activity – was measured in the conditioned media by its conversion of a colorless *p*-nitrophenylphosphate substrate (Sigma, cat. # P 5744) to a colored *p*-nitrophenol. The color change was measured at 405 nm in 3550-UV microplate reader (BioRad) and the amount of enzyme determined by comparison with a standard curve. All experiments were performed in duplicate.

**Hyaluronic acid production.** To determine the effect of SBD.4A on hyaluronic acid production, fibroblasts were seeded at 2500 cells/well in DMEM/5% FBS, exposed to SBD.4A, negative control (water) or positive control (bFGF), and grown for 6 days. At the end of the experiment cell culture conditioned media were collected for hyaluronic acid quantification, while cells were quantified by the sulforhodamine method as described above. HA was determined with the competitive ELISA kit from Echelon Biosciences (Salt Lake City, UT, cat. # K-1200) according to the manufacturer’s instructions. Briefly, samples of conditioned media were first mixed with the detector (the HA binding protein), then added to the HA ELISA plate for competitive binding. The colorimetric signal (hydrolysed pNPP) was detected at 405 nm with a BioRad 3550-UV microplate reader and was inversely correlated with the amount of HA present in the samples.

**Rat calvarial defect model**

**Preparation of the SBD.4A/PLG matrices.** In the 8 mg SBD.4A group, the composition of each sample was 8 mg SBD.4A + 8 mg PLG (poly-lactic/glycolide, Sigma) + 4 mg NaCl. Sixty mg PLG was heated, and the melted material was mixed thoroughly with 30 mg NaCl. The polymer/salt composite was then thoroughly mixed with 60 mg SBD.4A and the resulting material was cut at ~80 °C into seven pellets. Each of the devices was adjusted to 20 mg ± 1 mg, placed in an individual plastic vial, sealed in a plastic pouch and sterilized by e-beam sterilization.

In the 2 mg SBD.4A group, the composition of each sample was 2 mg SBD.4A + 2 mg PLG + 1 mg NaCl. The matrices were prepared as described above, using 15 mg PLG, 7.5 mg NaCl and 15 mg SBD.4A. Each of the devices was adjusted to 5 mg ± 1 mg.

**Testing of SBD.4A in the calvarial defect model.** This procedure was performed as reported before (Blom et al., 2001) with minor modifications. Briefly, 12 Wistar rats (Charles River Laboratories, Hollister, CA) were anesthetized and received one full thickness bone defect of 5 mm diameter on each side of the sagittal suture with a round dental bur (4.5 mm in diameter) on a slow handpiece, under constant irrigation with saline. On one side (control), the defects were filled with a slow release PLG matrix. On the opposite side, the defects were filled with PLG containing either 2 mg or 8 mg SBD.4A. After rats received SBD.4A and control PLG pellets as described above, the periosteum, connective tissue and skin were sutured back with 5-0 Vicryl resorbable suture and the animals were returned to cages for a 3 week period. After 21 days, the animals were killed by an overdose of CO₂, the calvaria were dissected with a round bur, fixed in 10% neutral formalin for 4 h, and then transferred into 70% ethanol. Calvaria were then sent to the laboratory of Phil Stashenko at Forsyth Institute in Boston and subjected to micro-computerized tomography (micro-CT; a fan-beam-type uCT 40, Scanco Medical AG, Bassersdorf, Switzerland) for bone micro-architecture analysis at a fee-for-service basis. The
defects were analysed using an initial scout view, selection of the examination volume, automatic positioning, measurement, off-line reconstruction and evaluation. For each sample, approximately 50 micro-tomographic slices with an increment of 17 μm were acquired. A constrained 3D Gaussian filter was used to partly suppress the noise in the volumes, and all samples were filtered using the same parameters for the filter width (1.0) and the filter support (1.0). Images were stored as 3D arrays with an isotropic voxel size of 17 μm. Morphometric parameters were computed using a direct 3D approach that does not rely on any assumptions about the underlying structure.

All animal protocols were approved by the Institute Animal Care and Use Committee (IACUC) of the Molecular Medicine Research Institute (MMRI) in Sunnyvale, CA, where the animal experiments took place. Animal experimentation was conducted in compliance with the U.S. Public Health Service Policy on the Human Care and Use of Laboratory Animals and all of the applicable provisions of the Animal Welfare Act, and other federal statutes and regulations relating to animals as guided by the U.S. Government Principles for the Utilization and Care of Vertebrate Animals in Testing, Research and Training (USDA).

**Statistical analysis.** Statistical significance of the observed differences in the average performance among groups for each experiment was determined by applying a two-tailed Student test (t-test). The differences were considered significant if the calculated t value was higher than the critical (tabulated) t value for confidence limit of 95% and 85% for the \textit{in vitro} and \textit{in vivo} experiments, respectively.

## RESULTS

In order to determine whether SBD.4A has potential for periodontal and/or bone regeneration \textit{in vivo}, first it was tested in a battery of relevant \textit{in vitro} tests. SBD.4A turned out to have a good stimulatory effect on MC3T3 osteoblast proliferation (Fig. 1A), but not differentiation (result not shown). Given the stimulatory activity of SBD.4A towards at least three cell types (endothelial cells, fibroblasts [Zhao \textit{et al.}, 2006] and osteoblasts) it was important to question whether SBD.4A could also exhibit pathogenic stimulation of cancer cells. Figure 1B shows that SBD.4A stimulates capillary endothelial cells, but not human breast cancer cells MDA MB231, even at an over 10 times higher concentration. SBD.4A was also tested on melanoma (B16F10) and prostate (PC3) cell lines and no stimulation observed [on the contrary, SBD.4A had a slight, but statistically significant (p < 0.05) inhibitory effect on these cell lines (maximal inhibition 15%, p < 0.05; results not shown)].

Hyaluronic acid (HA) is an extracellular matrix glycosaminoglycan (GAG), whose destruction by hyaluronidase-overexpressing periodontal pathogens contributes to the physiopathology of periodontitis [Scott \textit{et al.}, 1996; Last \textit{et al.}, 1985; Kesavalu \textit{et al.}, 2002]. Therefore, we set to determine whether SBD.4A can stimulate HA, by measuring the levels of this GAG in the culture media of

---

**Figure 1.** (A) SBD.4A strongly stimulated the proliferation of murine osteoblasts cell line MC3T3 grown in 96-well plates in 10% alpha minimum medium/90% DMEM/1% FBS. Number of tests (n) = 3. Error bars represent standard deviation. (B) Comparative effect of SBD.4A on the growth of capillary endothelial cells (BCE) and breast mammary epithelial adenocarcinoma cells (MDA-MB-231). \textit{In vitro}. Error bars represent standard deviation. SBD.4A concentrations for testing on endothelial cells were selected to show the active doses. SBD.4A concentrations for testing on adenocarcinoma cells were selected to demonstrate lack of effect even at over 10 times the dose active on endothelial cells.

---

Copyright © 2008 John Wiley & Sons, Ltd.


DOI: 10.1002/ptr
SBD.4A-treated fibroblasts. Basic fibroblast growth factor (bFGF), a well-known stimulator of HA (Asplund et al., 1993; Hirano et al., 2004; Shimabukuro et al., 2005), was used as a positive control. The results of the competitive HA ELISA show that SBD.4A indeed stimulates HA in the dose-dependent manner (Fig. 2). Furthermore, the comparison of panels A and B shows that there are two components to this stimulation, one being the increase in cell numbers and the other one – the amount of HA per cell.

Combined with previous findings on SBD.4A wound healing activities (Zhao et al., 2006), these data indicate that SBD.4A may have a beneficial effect on periodontal defect and/or bone regeneration in vivo. Therefore this assumption was assessed by applying SBD.4A in a slow-release matrix onto the rat calvarial defects.

First, the slow release matrix had to be selected. We first tried methylcellulose matrices and found that they can sustain SBD.4A release over a period of maximum 7 days (results not shown). Since the slow release of SBD.4A in our model had to occur over a longer period of time (21 days), methylcellulose was abandoned and PLG was employed instead. PLG matrix is an FDA-approved biodegradable material utilized for a large variety of active compounds. Furthermore, the release time of a water-soluble active material such as SBD.4A from PLG can be tuned to a 3 week period, compatible with our experimental protocol. Finally, PLG has been used in the calvarial defect model in the past (Zellin et al., 1998). The doses of SBD.4A have been based on the low and high end of therapeutic concentrations desired in the defect and on release rates of similar molecules (small, positively charged) from PLG matrices (Gonsalves et al., 1998). It is important to note that SBD.4A is a thermostable material and thus is suitable for the above-described procedure.

In order to characterize the SBD.4A bioactivity released from the PLG matrices, the SBD.4A/PLG (8 mg) matrix pellets were incubated in 1 mL saline for 3 weeks. At the end of each week, saline was collected and replaced. The bioactivity testing of the three saline samples from weeks 1, 2 and 3 shows that the SBD.4A material progressively diffused from the matrix while retaining its activity (Fig. 3). The relatively less vigorous fibroblast stimulation by the material released during the first week may be due to its excessive concentration (225 μg/mL final concentration in the cell culture medium), suggesting a bell-shaped dose-activity curve.

The effect of SBD.4A on calvarial bone regeneration was quantified by micro-computerized tomography (μCT), as described before (Balto et al., 2000; von Stechow et al., 2003). This quantification shows that SBD.4A has a stimulatory effect on the most representative parameter characterizing osteogenesis: BV/TV (bone volume/tissue volume fraction, Fig. 4). The SBD.4A-mediated stimulation is dose-dependent, no matter whether represented by the means (Fig. 4A) or medians (Fig. 4B). Figure 4A shows that the low concentration of SBD.4A (2 mg/pellet) enhanced bone regeneration only marginally (by 3%), while the high SBD.4A concentration (8 mg/pellet) resulted in over 17% stimulation. Figure 4B illustrates the fact that when medians were compared, low and high SBD.4A concentrations triggered 22% and 27% stimulation, respectively. Note the extremely reproducible defect size in both control groups.

**DISCUSSION**

The focus of this work was to assess the potential of SBD.4A, a multicomponent growth factor isolated from...
suggests that the cell-stimulatory effect of SBD.4A is of breast, prostate and melanoma cell lines. This result stimulatory effect on a panel of cancer cells composed of periodontal regeneration. Note that SBD.4A had no et al. (2006), which also occur in the earlier stages proliferation-dependant type I collagen output (Zhao et al., 2002). SBD.4A stimulated HA production by human fibroblasts by up to 55%. Higher levels of HA were due both to higher cell numbers and higher amounts of HA per cell. This result suggests that SBD.4A could play an important role in promoting homeostasis and inflammatory/wound healing responses mediated by human periodontal ligament (HPDL) cells, through the increase of HA production.

Animal experiments demonstrated that SBD.4A has bone generation-stimulatory activity, consistent with the preliminary in vitro results. This activity varies from 3% to 27% depending on the dose of SBD.4A and computation method (means vs medians). This is less than the increase reported for bFGF and statins (up to 46%; Mundy et al., 1999). However, this may be at least partially due to the release characteristics of the carrier. Indeed, typical PGA matrices, like ours, deliver a burst of the active material associated with the matrix (SBD.4A), then the release tapers off (see Fig. 3). The bone-stimulatory effect of SBD.4A in vivo could thus be potentially improved by increasing the load of the active or by modifying the carrier to obtain a more sustained release.

The difference between the means and medians may derive from the fact that the distribution in experimental groups is not Gaussian.

Finally, the superior bone generation effect by bFGF and statins is mitigated by the fact that bFGF treatment can cause dose-limiting hypotension (Laham et al., 2000), and that statin-triggered stimulation is transient, and associated with soft tissue inflammation (Thylin et al., 2002). In contrast, no inflammation was observed in this system (results not shown). It remains to be determined whether the bone gain induced by SBD.4A is more permanent than the statin-triggered one.

Taken together, the data presented here demonstrate the potential of SBD.4A for an adjuvant role in periodontal regeneration, as it stimulates cell types involved in periodontal regeneration and hyaluronic acid, in vitro, as well as bone regeneration in the calvarial model, in vivo. These findings make the case for the further development of SBD.4A into a multicomponent drug for the treatment of periodontitis, and possibly, other bone-destructive diseases.

Acknowledgements

This work was funded by the NIH SBIR Phase I grant 1R43DE015741-01A1 from the National Institute of Dental and Craniofacial Research. We would like to thank Anjani Attili for expert technical support, as well as Drs Phil Stashenko and Richard Niederman from Forsyth Institute (Boston, MA) for CT measurements and helpful discussions.


Copyright © 2008 John Wiley & Sons, Ltd.

*Phytother. Res. (2008)*

DOI: 10.1002/ptr