Secretoneurin, a Neuropeptide, Enhances Bone Regeneration in a Mouse Calvarial Bone Defect Model

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Abstract

BACKGROUND: This study investigates the effects of a neuropeptide, secretoneurin (SN), on bone regeneration in an experimental mouse model.

METHODS: The effects of SN on cell proliferation, osteoblast marker genes expression, and mineralization were evaluated using the CCK-8 assay, quantitative reverse transcriptase polymerase chain reaction (RT-PCR), and alizarin red S staining, respectively. To examine the effects of SN on bone regeneration *in vivo*, bone defects were created in the calvaria of ICR mice, and 0.5 or 1 µg/ml SN was applied. New bone formation was analyzed by micro-computed tomography (micro-CT) and histology. New blood vessel formation was assessed by CD34 immunohistochemistry.

RESULTS: SN had no significant effect on proliferation and mineralization of MC3T3-E1 cells. However, SN partially induced the gene expression of osteoblast differentiation markers such as runt-related transcription factor 2, alkaline phosphatase, collagen type I alpha 1, and osteopontin. A significant increase of bone regeneration was observed in SN treated calvarial defects. The bone volume (BV), BV/tissue volume, trabecular thickness and trabecular number values were significantly increased in the collagen sponge plus 0.5 or 1 µg/ml SN group ($p < 0.01$) compared with the control group. Histologic analysis also revealed increased new bone formation in the SN-treated groups. Immunohistochemical staining of CD34 showed that the SN-treated groups contained more blood vessels compared with control in the calvarial defect area.

CONCLUSION: SN increases new bone and blood vessel formation in a calvarial defect site. This study suggests that SN may enhance new bone formation through its potent angiogenic activity.

Keywords Neuropeptide · Secretoneurin · Bone regeneration · Bone

1 Introduction

Bone repair is a complex process that leads to new bone formation through cellular and molecular processes. It is regulated by multiple biological factors, such as the transforming growth factor- β (TGF- β) superfamily members and proinflammatory cytokines [1]. The bone repair and fracture healing processes consist of several overlapping stages including inflammation and angiogenesis [2]. Angiogenesis is a dynamic and complex physiological process that plays a central role in repairing damaged bone by supplying sufficient nutrients, oxygen, calcium, and

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phosphate. It also serves as route to deliver mesenchymal stem cells, which differentiate into osteoblast and chondrocytes at the defect site [3–6].

Recent studies have demonstrated that enhancing angiogenesis can accelerate bone regeneration [7]. However, inappropriate blood vessel supply is a major cause of delayed union or non-union formation during fracture healing [8]. Moreover, inhibition of angiogenesis prevents the formation of callus and periosteal woven bone during fracture healing in rats [9]. Angiogenesis is carefully regulated by angiogenic factors [4, 10]. Importantly, the local application of angiogenic factors alone or combination with biomaterials to sites with bone defects has been shown to increase new bone formation [11, 12].

Secretoneurin, a 33 amino acid neuropeptide, is produced by proteolytic cleavage of secretogranin II, a storage vesicle and member of the chromogranin/secretogranin family. It is expressed and released in the brain, adrenal medulla, endocrine tissues, primary sensory C-fibers, and sympathetic adrenergic neurons of the peripheral nervous system [13–16]. SN gene therapy was shown to accelerate skin wound closure in a diabetic mouse model by enhancing capillary and arteriole density [17]. Additionally, it has been reported that SN exerts an antiapoptotic effect and increases proliferation in endothelial cells. It also possesses potent angiogenic properties and induces postnatal vasculogenesis [18, 19]. SN also exhibits angiogenic activity similar to other neuropeptides, such as substance P (SP) and neuropeptide Y, *in vitro* and *in vivo* [20]. Recently, it has been demonstrated that SN enhances the mobilization and homing of bone marrow mesenchymal stem cells (BMSCs) to the brain [21]. However, it remains unclear whether SN can be used as a therapeutic agent for bone regeneration.

We hypothesized that the local application of the SN angiogenic neuropeptide, which has shown to enhance angiogenesis, mobilization, and homing of BMSCs, could induce calvarial bone regeneration. For this study, a calvarial defect was created in murine calvaria and two different doses of SN were applied locally using a collagen sponge carrier.

2 Materials and methods

2.1 Cell culture and induction of osteoblast differentiation

MC3T3-E1 cells were purchased from the ATCC (Manassas, VA, USA; CRL-2593). The cells were cultured in alpha-minimum essential medium (α -MEM) without ascorbic acid (Welgene, Gyeongsan, Korea) and contained 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin) and incubated at 37 °C in the presence of

$\leq 5\%$ CO₂. The cell culture media for positive controls and experimental groups was as follows: α -MEM containing 10% FBS, 10 mM β -glycerophosphate, and 50 μ g/mL ascorbic acid to induce osteoblast differentiation in the positive control group. The same medium with a serial dilution of SN (50 ng/ml, 10 ng/ml and 1 ng/ml) was used for the experimental groups. The SN peptide which was obtained from AnaSpec Inc. (Fremont, CA, USA), was dissolved in phosphate buffered saline (PBS) and this solvent was used as a control.

2.2 Cell proliferation assay

The effect of SN on MC3T3-E1 cell viability was evaluated using a CCK-8 assay kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The MC3T3-E1 cells were seeded into 96-well plates at 7×10^3 cells per well. After 24 h, cells were treated with three different concentrations of SN (1, 10 and 50 ng/ml) and cultured for 24, 48, and 72 h. Three replicate wells per sample were prepared. After that, the original medium was carefully removed, and 100 μ L of fresh medium plus 10 μ L CCK-8 solution were added to each well, and the plates were incubated at 37 °C, under 5% CO₂ atmosphere for 2 h. The absorbance at 450 nm was measured for each well using a microplate reader (BioRad, Hercules, CA, USA).

2.3 Quantitative RT-PCR

Osteoblast differentiation in response to SN was examined by measuring the expression of osteoblast differentiation marker genes by quantitative RT-PCR. The cells were incubated in 24-well plates at a density of 1×10^5 cells per well. After a 24 h incubation, all the groups were cultured in osteogenic medium. The experimental groups were also exposed to three different concentrations of SN (1, 10 and 50 ng/ml). The media was changed every three days. The cells were then harvested on the 7th and 14th day following osteogenic differentiation with or without SN. Total RNA was extracted using TRIzol-solution (BSK-BIO, Daegu, Korea) according to the manufacturer's instructions. Complementary DNA was prepared from 1 μ g of RNA, using Superscript II (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR was performed using a Light Cycler 1.5 system (Roche Diagnostics, Rotkreuz, Switzerland) with SYBR® Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan). *Gapdh* was used as a housekeeping gene. The forward and reverse primers used for RT-PCR are listed in Table 1.

2.4 Alizarin red S staining

The mineralization of MC3T3-E1 cells resulting from SN treatment was measured by alizarin red S staining. The cell

Table 1 Primers sequences used for quantitative RT-PCR

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>Gapdh</i>	ATG ACA TCA AGA AGG TGG TG	CAT ACC AGG AAA TGA GCT TG
<i>Runx2</i>	ACT CTT CTG GAG CCG TTT ATG	GTG AAT CTG GCC ATG TTT GTG
<i>Alp</i>	AAC CCA GAC ACA AGC ATT CC	GAG AGC GAA GGG TCA GTC AG
<i>Colla1</i>	CCT AAT GCT GCC TTT TCT GC	ATG TCC CAG CAG GAT TTG AG
<i>Opn</i>	AGC AAG AAA CTC TTC CAA GCA A	GTG AGA TTC GTC AGA TTC ATC CG

culture methods were the same as described above. After 28 days, the original culture medium was discarded and MC3T3-E1 cells were gently washed twice with PBS, followed by fixation in 70% ethyl alcohol for 10 min at room temperature. The cells were then rinsed with distilled water. Subsequently, the cells were stained with alizarin red S staining solution (2%, pH 4.2) for 10 min, washed with distilled water, and allowed to dry overnight. Finally, the cells were observed and photographed using microscopy (Olympus SZX-TR30, Tokyo, Japan).

2.5 Animal model and surgical procedure

Animal experiments were performed in accordance with the guidelines approved by Kyungpook National University (No. 2018–0153). Fourteen ICR mice (6 weeks old) were divided into three groups: collagen sponge plus PBS (control) ($n = 4$), collagen sponge plus 0.5 $\mu\text{g}/\text{ml}$ SN ($n = 5$), and collagen sponge plus 1 $\mu\text{g}/\text{ml}$ ($n = 5$). Collagen sponge was used as a carrier since it is a natural polymer suitable to local delivery and retains biomolecules to the defect site [22]. Animals were anesthetized by intraperitoneal injection of a mixture containing tribromoethanol (Sigma Aldrich, St Louis, MO, USA) (22 $\mu\text{l}/\text{g}$). The hair over the skull was shaved and the underlying skin was aseptically prepared using a providone/betadine scrub. Then, a sagittal skin incision was made over the scalp from the frontal to the occipital bone and the skin flap including the periosteum. Calvarial defects were created in the left parietal bone using a trephine (3 mm diameter) bur (MCTBIO, Yongin, Korea) with a slow dental handpiece (Surgic XT, Nakanishi, Japan). Calvarial defect sites were continuously irrigated with PBS during drilling to wash out any bone fragments. After removal of the trephined calvarial disk, a 4 mm diameter collagen sponge (Sponge-Col®, San Diego, CA, USA) and SN neuropeptide were applied to the defect site. Finally, the periosteum and skin were closed and sutured with surgit 5–0 (AILEE Co., Busan, Korea).

2.6 Micro-CT evaluation

The mice were sacrificed 8 weeks following surgery by cervical dislocation. The calvarial bone was excised,

trimmed, and fixed in 10% neutral buffered formalin solution at room temperature overnight. The calvaria were transferred to PBS and stored at 4 °C until micro-CT analysis. The specimens were scanned using a Skyscan 1272 Scanner (Bruker-microCT, Konich, Belgium) (X-ray voltage 70 kV, 142 μA anode current, 0.5 mm Aluminum filter, isotropic voxel size 10 μm , and 537 ms exposure time). After standardized reconstruction using CTvol software, the datasets were analyzed using CTAn software (Bruker-microCT) [23]. The cylindrical volume of interest was defined as 3 mm in diameter and 0.3 mm in height in order to include all of the new bone formation in the calvarial defect site.

2.7 Histologic analysis

Following micro-CT, the specimens were decalcified using 0.5 M EDTA for 2 weeks. After decalcification, the specimens were paraffin-embedded. Histological Sects. (6 μm in thickness) were prepared using a microtome (Leica, Nussloch, Germany). The sections were stained with haematoxylin and eosin (H&E). Images of the stained sections were obtained by microscopy (Leica, Wetzlar, Germany). The newly formed bone at defect site was quantified from the section stained with H&E using iSolution software (Daejeon, Korea) and the result was described as percentage of area of new bone (area of new bone divided by area of the defect site) [24]. At least two samples were considered for each sample.

2.8 Immunohistochemistry

Briefly, after deparaffinization and hydration of the paraffin sections, heat-mediated antigen retrieval was performed in 10 mM sodium citrate buffer, pH 6.0, containing 0.05% Tween-20 (Junsei Chemical Co., Ltd., Tokyo, Japan) and incubated with anti-CD34 polyclonal antibody (Boster Biological Technology, Pleasanton, CA, USA) at a 1:500 dilution overnight. Goat anti-rabbit IgG (Santa Cruz Biotechnology Inc., Dallas, TX, USA) was used as a secondary antibody (1:500 dilution) followed by color development using 3, 3'-diaminobenzidine tetrahydrochloride. As a negative control, PBS was substituted for the primary antibody. A qualitative analysis was performed using light

microscopy (Leica). Finally, histological sections were examined to quantify the new blood vessels at defect site. Blood vessels were manually counted and CD34 positive structure was taken to indicate a blood vessel [25, 26]. At least two samples were considered for each sample.

2.9 Statistical analysis

All results are expressed as the mean $\pm$ SEM. Statistical comparisons between two groups were done by a Student's t-test. Probability values < 0.05 or < 0.01 were considered statistically significant. All experiments were repeated at least in triplicate.

3 Results

3.1 Effect of SN on proliferation of MC3T3-E1 cells

The effect of SN on the proliferation of MC3T3-E1 cells was determined by a CCK-8 assay at different concentrations of SN (1, 10 and 50 ng/ml), which were determined in a previous experiment [27]. The cells were incubated for 24, 48, and 72 h following SN treatment. The results revealed that cellular proliferation was not significantly different between the control group and SN treated groups at 24, 48, and 72 h (Fig. 1).

3.2 Effect of SN on osteoblast differentiation and mineralization of MC3T3-E1 cells

The effect of SN on mineralization was determined using alizarin red S staining to measure calcium deposition at 4 weeks after treatment. The results indicated that there was no significant difference in calcium deposition between the SN-treated groups and the control group (Fig. 2). However, expression levels of the osteoblast

Fig. 1 Effect of SN on the proliferation of MC3T3-E1 cells. The cells were treated with different concentrations (control, 1, 10 and 50 ng/ml) of SN for 24, 48 and 72 h, and cell viability was assessed by CCK-8

differentiation markers, including Runx2, Alp, Col1a1, and Opn, were partially increased by SN at days 7 and significantly increased at days 14 with 50 ng/ml SN treatment ($p < 0.05$) during osteoblast differentiation (Fig. 3).

3.3 Effect of SN on the regeneration of the calvarial defect

3.3.1 Micro-CT evaluation

To investigate the effect of SN on bone defect repair *in vivo*, the newly formed bone at the calvarial defect site was assessed using micro-CT. Representative micro-CT images of the mouse calvaria at 8 weeks following creation of the defect are shown in Fig. 4A. The bone morphometric parameters of the defect site that were analyzed include bone volume (BV), BV/tissue volume (TV), trabecular thickness (Tb.Th), and trabecular number (Tb.N). At 8 weeks post-surgery, a significant increase in BV, BV/TV, and Tb.N was observed in the collagen sponge plus 0.5 μ g/ml SN ($p < 0.01$) group compared with the control group. However, no marked difference in Tb.Th was observed between the collagen sponge plus 0.5 μ g/ml SN group and control group. In addition, BV, BV/TV, Tb.Th, and Tb.N values were significantly increased in the collagen sponge plus 1 μ g/ml SN groups ($p < 0.01$) compared with the control group. As expected, the BV, BV/TV, Tb.N, and Tb.Th values from the collagen sponge plus 1 μ g/ml SN group were the highest compared with the other groups (Fig. 4B).

3.3.2 Histologic analysis

H&E staining revealed that at 8 weeks after defect creation, dense and large amounts of fibrous tissue were observed in the control group. At higher magnification, osteoids were also identified. As expected, in SN treated groups, in addition to osteoids, new bone formation was observed at the defect margins (arrow heads). The collagen sponge plus 1 μ g/ml SN group exhibited greater amounts of newly formed bone compared with the collagen sponge plus 0.5 μ g/ml SN group. However, the control group had shown a minimal new bone formation (Fig. 5A). The mean $\pm$ standard deviation (SD) of new bone area (%) in histological sections of 1 μ g/ml, 0.5 μ g/ml SN treated and control groups were $7.0 \pm 2.2\%$ ($p < 0.01$), $4.2 \pm 1.7\%$ ($p < 0.05$) and $1.4 \pm 0.3\%$, respectively (Fig. 5B).

3.3.3 Immunohistochemical analysis of CD34

To evaluate the angiogenic effect of SN in the calvarial defect area, the angiogenic marker, CD34, was assessed by immunohistochemical staining. There was virtually no obvious positive staining for CD34 in the control group. However, significant positive staining for CD34 was

Fig. 2 Effect of SN on mineralization of MC3T3-E1 cells. After treatment with various concentrations (control, 1, 10 and 50 ng/ml) of SN for 28 days, mineral deposition in cells was measured by alizarin red S staining. Scale bar = 1 mm

Fig. 3 Effect of SN on the expression of osteoblast differentiation marker genes in MC3T3-E1 cells. The cells were treated with different concentrations (control, 1, 10 and 50 ng/ml) of SN for 7 and 14 days. The mRNA expression level of *Runx2*, *Alp*, *Col1a1* and *Opn* was assessed by quantitative RT-PCR. *Gapdh* was used as a

reference. *Runx2*, runt-related transcription factor 2; *Alp*, alkaline phosphatase; *Col1a1*, collagen type I alpha 1 and *Opn*, osteopontin. An asterisk represents significant difference between groups. * $p < 0.05$ as compared with control

observed in the SN treated groups (arrow heads). Increased positive staining was observed in the collagen sponge plus 1 $\mu\text{g/ml}$ SN group compared with the collagen sponge plus 0.5 $\mu\text{g/ml}$ SN group (Fig. 6A). The

mean $\pm$ SD of blood vessels number in 1 $\mu\text{g/ml}$, 0.5 $\mu\text{g/ml}$ SN treated and control groups were 7 ± 1 ($p < 0.01$), 5 ± 1 ($p < 0.05$) and 2 ± 1 , respectively (Fig. 6B).

A **B**

Fig. 4 Micro-CT scanning and quantitative analysis of calvarial defects at 8 weeks post-surgery. The treatment groups include control, collagen sponge plus 0.5 $\mu\text{g/ml}$ and collagen sponge plus 1 $\mu\text{g/ml}$. **A** Three-dimensional reconstructed micro-CT images of newly formed bone at the calvarial defect sites at 8 weeks after surgery. **B** Quantitation of BV, BV/TV, Tb.N and Tb.Th from newly formed bone. BV: bone volume; BV/TV: the ratio of mineralized bone volume to the defect tissue volume; Tb.N: the trabecular number; Tb.Th: the trabecular thickness of newly formed bone. Asterisks represent significant difference between groups. $**p < 0.01$ as compared with control

4 Discussion

In the present study, we demonstrated for the first time that SN treatment accelerates bone regeneration in a murine calvarial defect model. Bone regeneration is a complex physiological process involving not only osteogenesis, but also angiogenesis [28]. It has been also demonstrated that angiogenesis precedes osteogenesis during bone repair. The process of angiogenesis is regulated by several molecules including growth factors and peptides. In animal

Fig. 5 H&E staining of the calvarial defect area at 8 weeks after surgery. The treatment groups include control, collagen sponge plus 0.5 $\mu\text{g/ml}$ and collagen sponge plus 1 $\mu\text{g/ml}$. **A** The arrow heads indicate the newly formed bone. Upper panels (low magnification, $\times 16$); scale bar = 1 mm and lower panels (high magnification

of boxed regions, $\times 100$); scale bar = 200 μm . **B** Percentage area of new bone at defect site. Asterisks represent significant difference between the groups. $*p < 0.5$ and $**p < 0.01$ as compared with the control group

A **B**

Fig. 6 Immunohistochemical staining of CD34 at calvarial defect sites at 8 weeks after surgery. The treatment groups include control, collagen sponge plus 0.5 µg/ml and collagen sponge plus 1 µg/ml. **A** The arrow heads indicate CD34 positive blood vessels. Upper panels (low magnification, × 100); Scale bar = 200 µm and lower

panels (high magnification of boxed regions, × 400); scale bar = 50 µm. **B** Number of new blood vessels at defect site. Asterisks represent significant difference between the groups. * $p < 0.5$ and ** $p < 0.01$ as compared with the control group

models, the administration of angiogenic factors, such as vascular endothelial growth factor, to the bone defect has been shown to induce neovascularization and bone regeneration [4–7, 10, 29–31].

In vitro experiments showed that SN had no significant effect on proliferation and mineralization in MC3T3-E1 cells. Osteoblast differentiation marker genes, including Runx2, Col1a1, Alp, and Opn, are widely used for evaluating osteoblast differentiation [32–34]. The expression of these marker genes was tended to increase by SN. Furthermore, alizarin red S staining confirmed that SN was unable to stimulate mineralization in MC3T3-E1 cells.

These results indicate that SN may partially exert direct effects on osteoblast differentiation in MC3T3-E1 cells. Rather, SN may affect bone regeneration because it possesses angiogenic activity and induces mobilization and homing of BMSCs [18, 21, 27]. The homing of BMSCs is a critical step in bone repair and BMSCs can differentiate into osteoblasts to accelerate bone regeneration [35, 36]. Therefore, we hypothesized that SN may enhance bone regeneration by promoting angiogenesis in the defect area and we established a calvarial defect animal model.

Micro-CT evaluation revealed that the SN groups displayed a higher amount of new bone formation compared

with the control group at 8 weeks following surgery. The bone morphometric (BV, BV/TV, Th.Tb and Th.N) values for newly formed bone and the images acquired from the representative specimens confirmed a positive effect of SN on bone regeneration (Fig. 4). In addition, histologic analysis complemented the micro-CT findings of bone formation within the defect area. The collagen sponge plus 1 µg/ml SN group displayed maximal newly-formed bone near the defect sites followed by the collagen sponge plus 0.5 µg/ml SN group, whereas osteoid formation was more pronounced in the control group (Fig. 5). These findings are consistent with our hypothesis that local application of SN promotes bone regeneration. Interestingly, other neuropeptides such as SP and vasoactive intestinal peptide (VIP) have been shown to induce proliferation and osteoblast differentiation of BMSCs or osteoblast progenitor cells. These differences may arise from expression of the neurokinin 1, a receptor for SP and activation of the Wnt/ β -catenin signaling pathway by both SP and VIP in BMSCs or pre-osteoblast progenitor cells [37–40]. In contrast, the receptor for SN, which remains unknown, may not be highly expressed in MC3T3-E1 cells. Moreover, immunohistochemical staining for CD34 in the calvarial defect area also revealed that SN promotes new blood vessel formation (Fig. 6), which may facilitate new bone formation. This finding is consistent with previous studies which have shown that SN exhibits a potent angiogenic effect and induces neovascularization in the mouse cornea [18, 19].

Based on our experimental results, we suggest that SN may regulate angiogenesis and partial osteogenesis in the bone defect area and facilitate new bone formation. There are also reports supporting angiogenic peptides in the acceleration of bone regeneration by promoting angiogenesis [41]. Importantly, several previous studies demonstrated that the combined use of angiogenic and osteogenic growth factors facilitate new bone formation and neovascularization compared with either factor alone [42, 43]. Therefore, the application of SN in combination with other biomolecules can induce osteogenesis and may significantly improve bone regeneration in injured tissues.

In summary, the results this study have demonstrated that the local application of SN significantly increased bone regeneration in a mouse calvarial defect model. This could be attributed to its potent angiogenic activity.

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performed the experiments and analyzed the results; F. A., H. J. I., Y. C. B. and E. K. P. analyzed the data and revised the manuscript; and E. K. P. supervised the project.

Compliance with ethical standards

Conflict of interest The authors have no financial conflicts of interest.

Ethical statement Animal experiments were performed in accordance with the guidelines approved by Kyungpook National University (No. 2018–0153).

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