CHARACTERISTICS OF NEWLY FORMED BONE DURING GUIDED BONE REGENERATION:
ANALYSIS OF CBFA-1, OSTEOCALCIN, AND VEGF EXPRESSION

Shiho Tanaka, DDS, PhD; Kenichi Matsuzaka, DDS, PhD; Daisuke Sato, DDS; Takashi Inoue, DDS, PhD

This study investigated the expression of core-binding factor alpha-1 (cbfa-1), osteocalcin, and vascular endothelial growth factor (VEGF) relative to new bone formation during guided bone regeneration; cbfa-1 is a prerequisite transcription factor for osteoblastic differentiation. Osteocalcin, a bone-specific extracellular matrix protein, is a marker of mature osteoblasts, whereas VEGF, a mitogen for endothelial cells, is a polypeptide thought to stimulate new blood vessel formation. Membranes (expanded polytetrafluoroethylene) were applied to defects created in the left tibiae of rats, while right tibial defects remained uncovered as a control group. Animals were killed 6, 8, or 10 days later. The cbfa-1 was detected by immunohistochemistry, and reverse transcriptase-polymerase chain reaction was used to detect osteocalcin and VEGF. The ratio of cbfa-1 positive cells in experimental bone defects was higher than in the control group. Osteocalcin mRNA expression increased gradually in the control group but significantly in the experimental group over time. The VEGF mRNA expression in the experimental group at 10 days was significantly lower than in the control group. These findings suggested that osteogenic cells differentiated into osteoblasts in the membrane-covered defects and that the bone healing process would be completed at an early stage.

Key Words: guided bone regeneration; cbfa-1; osteocalcin; VEGF; immunohistochemistry; mRNA expression

INTRODUCTION

Guided bone regeneration (GBR) is a method that facilitates the repair of bone defects. The principle of GBR was originally based on guided tissue regeneration methods, which isolated bone defects from gingival connective tissues and created an exclusive space into which only cells from the surrounding bone could migrate. Becker et al\(^1\) compared new bone formation with and without the GBR method and found that GBR achieved repair of bone defects in the same manner as bone grafts or bone substitutes.

Our group examined the characteristics of new bone formation during GBR by morphologic analysis.\(^2\) In that study, the ratios of proliferating cells and newly formed bone in bone defects with membranes were higher than those in the controls. We concluded that the GBR method prevents undesirable cells from possibly invading the wound area because of the...
CHARACTERISTIC OF BONE DURING GBR

barrier membrane and because it favors the proliferation of defined cells to promote wound healing with newly formed bone. Furthermore, the newly formed bone generated with GBR matures more rapidly than it does in the absence of the membrane. However, that conclusion was a matter for speculation and the results did not prove it.

Although there are many kinds of GBR membrane, expanded polytetrafluoroethylene (e-PTFE) is the most popular membrane; it is nonabsorbable and has a matrix of PTFE nodes and fibrils on a microstructure that can be varied in porosity to address the clinical and biological requirements of its intended applications.3

Core-binding factor alpha-1 (cbfa-1) is known as a transcriptional activator of osteoblast differentiation.4,5 Detecting cbfa-1 in bone defects with or without membranes would show that the proliferating cells noted in our previous study differentiated to osteogenic cells. Further, osteogenic protein and vascular endothelial growth factor (VEGF) play important roles in the wound healing of bone. VEGF is required for the development of endothelial cell-based microvascularization and microcirculation, not only for soft tissue wound healing but also for preserving structure and regeneration of bone.6,7

The purpose of this study was to further investigate the characteristics of new bone formation during GBR as it relates to cbfa-1, osteocalcin, and VEGF.

MATERIALS AND METHODS

Surgical procedures and animals

Twenty-four male Sprague-Dawley rats (250 g body weight) were used in this study. Nine of those rats were used for morphologic observation and analysis, and the other 15 were used to examine mRNA expression. Animals were anesthetized by sodium thiopental (Pabonal, Tnbrane Co Ltd, Osaka, Japan), an incision in the skin was made along the lateral aspect of each leg, and the muscles and periosteum were retracted to expose the lateral aspect of the tibia. Defects approximately 6 mm by 2.5 mm were generated in the middle region of each tibia using a round burr mounted in a dental handpiece cooled with phosphate-buffered saline (PBS). While creating defects, bone marrow and periosteum were also removed. After defects were created, astringion was done using gauze. The e-PTFE membranes (Gore-Tex, WLGore, Flagstaff, Ariz) were cut to the appropriate size and rolled around the defect on the left leg of each animal. The right tibial defect remained uncovered as for a control. After the operation, muscle and skin were sutured to hold the membrane.

Morphologic observation

Rats were killed with an overdose of sodium pentobarbital at 6, 8, or 10 days after the operation (3 rats per day). Each tibia was removed and was fixed in 4% paraformaldehyde for 2 days and then demineralized with 10% ethylenediaminetetraacetic acid for 14 days at 4°C before embedding in paraffin. Sections were deparaffinized with xylene, rehydrated in 100% alcohol, and washed in distilled water. Serial sections were cut and stained with hematoxylin-eosin using routine procedures. Endogenous peroxidase activity was blocked by incubating sections with 3% H2O2 in methanol for 30 minutes. To prevent nonspecific binding, sections were incubated in a 10% serum solution for 30 minutes in a 100% humidity chamber. Sections were stained immunohistochemically with an anti-cbfa-1 antibody (1:400, PEBP2αA [S-19], Santa Cruz Biotechnology, Santa Cruz, Calif). Slides were washed in PBS and then incubated with the secondary antibody for 30 minutes in a humidity chamber. After washing in PBS (5 minutes, 3 times), sections were stained with 3,3'-diaminobenzidine for 5 minutes, washed in distilled water, counterstained in hematoxylin, and coverslipped.

Analysis of cbfa-1 positive cell ratios

Defects were divided into 2 or 3 areas, as described by Matsuzaka et al.2 Briefly, bone sides examined were areas adjacent to the compact bone edge, the central sides longitudinally, and the upper and lower sides laterally (Figure 1). The ratio of cbfa-1 positive cells was calculated for each side, and results are expressed as percentage of cbfa-1 positive cells ± SD per mm.2

Quantitative RT-PCR using the LightCycler

For evaluation of osteocalcin and VEGF mRNA levels, rats were sacrificed on days 6, 8 or 10 (5 rats each time point) after the operation, with an overdose of sodium pentobarbital. The tibiae were exposed, and tissues in the cavity were removed mechanically using a dental spoon excavator and dental chisel into an Eppendorf tube. For the quantitative analysis of osteocalcin and VEGF mRNA expression, total RNA was extracted using the Isogen reagent (Nippon Gene, Japan) according to the manufacturer’s instructions. Briefly, cells were homogenized and solubilized in Isogen/chloroform solution at 4°C. Supernatants were obtained following centrifugation at 12 000g for 20 minutes at 4°C. The precipitates were recovered following decantation and were washed with 75% ethanol. The RNA pellets
were then dissolved in RNase-free water, and were preserved at \(-20^\circ\text{C}\) until used. Using the extracted RNA as a template, reverse transcription reactions were conducted with an reverse transcriptase-polymerase chain reaction kit (RNA-PCR kit version 2.1, Takara Biomedicals, Japan) to synthesize cDNA. Quantitative polymerase chain reaction (PCR) was then conducted using primers for VEGF and osteocalcin, HSP70 with a LightCycler (Roche Diagnostics, Mannheim, Germany) using the double-stranded DNA dye SYBR Green I (Roche Diagnostics).

Quantification was performed by comparing the levels obtained with standard samples. In the present study, the concentrations of cDNA in the unstimulated samples were 0.2, 0.5, 1.0, and 2.0 \(\mu\text{L}\). The PCR conditions used in the LightCycler were 40 cycles, while PCR conditions were 95°C for 10 seconds, 60°C for 5 seconds, and 72°C for 12 seconds. After the PCR amplification, melting curve analyses were also performed to confirm the absence of the primer dimer in the PCR products. The ratios of osteocalcin (199 base pair [bp]; forward; GGT GCA AAG CCC AGC GAC TCT, Reverse; GGA AGC CAA TGT GGT CCG CTA) and VEGF (203 bp; Forward; TAC CAG CGC AGC TAT TGC CGT, Reverse; TTT GGT GAG GTT TGA TCC GCA TG) mRNA expressions were adjusted by the value of the housekeeping gene \(\beta\)-actin mRNA (260 bp; forward; CCT GTA TGC CTC TGG TCG TA, Reverse; CCA TCT CTT GCT CGA AGT CT). Four independent experiments were performed for all assays of cell behavior; 2 specimens of each sample type were used in each experiment.

**Statistical analysis**

Data from each experiment were statistically analyzed and the assumption of homogeneity of variances and normal distribution of errors were tested for the response variables evaluated. One-way analysis of variance and a multiple comparison test (Scheffe’s test) were used to compare changes over time \((P < .05)\), and Student’s \(t\) test was used to compare various time periods \((P < .05)\).

**Results**

**Morphologic observations**

Hematoxylin-eosin staining in this study showed the similar results of Matsuzaka et al.\(^2\) A fibrous connective tissue was observed close to the membrane at 6 days, but new bone was filled up to the membrane at 8 and 10 days in the experimental group. In the control group, muscle and fibrous connective tissue invaded into the defect.

We observed cbfa-1 positive cells in the fibrous connective tissue, around the bone tissue, and in the newly formed woven bone, but not in the mature bone tissue. In the experimental group, cbfa-1 positive cells were never detected at the outer area of the membrane at any time period, but many cbfa-1 positive cells remained in the defect at 6 days. At 8 and at 10 days, cbfa-1 positive cells were observed around newly formed bone. At 6 days, cbfa-1 positive cells were found at the lower area in the control group but were observed diffusely in the upper and lower areas in the experimental group (Figure 2a and b). Although at 10 days, cbfa-1 positive cells were observed at both the upper and lower areas in the control group, the number of cbfa-1 positive cells in the experimental group was reduced (Figure 2c and d). In addition to those immunohistochemical observations, many capillary vessels were observed in the experimental group at 10 days and granulation tissue was seen in the upper area.
Ratios of cbfa-1 positive cells

In the bone side and central side, the ratio of cbfa-1 positive cells in the experimental group at 6 days was the highest; it decreased with time after that, but the ratios in the control group were not significantly different at any time period (Figures 3 and 4).

In the upper side and lower side, the cbfa-1 positive cell ratio was the highest in the experimental group at 6 days and was the highest in the control...
group at 8 days (Figure 5). In the lower side, the cbfa-1 positive cell ratio was the highest in the experimental and control groups at 6 days and decreased after that with time (Figure 6).

**mRNA expression**

Expression of osteocalcin mRNA in the control group gradually increased with time, but in the experimental group it significantly increased at 8 and 10 days (Figure 7). The expression of VEGF mRNA in both groups were not significantly different at 6 and 8 days, but it was significantly different at 10 days (Figure 8).

**Discussion**

Osteoblasts are cells of mesenchymal origin that, once they terminally differentiate, produce most of the proteins present in the bone extracellular matrix; they also control the mineralization of this extracellular matrix. Progress in understanding osteoblast differentiation has been hampered by the small number of molecular markers truly specific for osteoblasts and by the absence of morphologic features distinguishing these cells from fibroblasts. It is known that pre-osteoblasts as progenitor cells secrete type I collagen, alkaline phosphatase, and osteonectin and that secretion of osteocalcin and bone sialoprotein by juvenile osteoblasts produces bone tissue. On the other hand, cbfa-1 is a required transcription factor produced when pre-osteoblasts differentiate to osteoblasts.

Matsuzaka et al reported that the ratios of newly formed bone in the space created by the barrier membrane were higher than in the control groups at all experimental time periods, although the ratios of proliferating cells in those spaces were not significantly different between both groups. They concluded that only cells that form bones can proliferate in the space created by the e-PTFE membrane. However, that remained a matter of speculation, so detecting cbfa-1 would help distinguish if they were osteogenic cells. The results of this study, in which the ratio of cbfa-1 positive cells in the upper side of the experimental group is higher than the control group, support the previous report. The ratio of cbfa-1 positive cells in the upper side of experimental group significantly decreased from 6 to 8 days. This also means that the osteogenic cells differentiate into the osteoblasts in the bone defect at an early stage of wound healing. In the lower side, deterioration of the ratios of cbfa-1 positive cells with time in control groups is similar to the experimental group. This means that the lower side is unaffected by the membrane. Further, the ratio of cbfa-1 positive cells on the bone side of the experimental group, which is higher than the control group, also supports the
previous study. In the upper side of the control group, however, proliferating cells may not be osteogenic cells but rather cells that exist at the stage of soft tissue wound healing.

Osteocalcin is known as a protein involved in osteogenic differentiation, and it is secreted by osteoblasts after bone calcification starts.\(^5,10\) Increased osteocalcin mRNA means cells are differentiating from preosteoblasts to osteoblasts. Osteocalcin mRNA in the control group gradually increased with time, but in the experimental group it increased significantly. This suggests that GBR accelerates osteogenic cell differentiation.

On the other hand, VEGF is known as an angiogenic growth factor that elicits cellular responses to hypoxia.\(^11,12\) Induction of VEGF has also been investigated in rat astrocytes and in the heart during hypoxic conditions.\(^13,14\) Development of endothelial cell-based microvascularization and microcirculation is undoubtedly crucial for the preservation of structure and regeneration of bone.\(^15\) In this study, expression of VEGF mRNA in both groups was not significantly different at any time period, but it was significantly higher in the control group than in the experimental groups at 10 days after the operation. Matsuzaka et al\(^2\) reported that the ratio of newly formed bone in the defect created by the barrier membrane was increased at an early stage. This means bone healing with GBR might be completed at 10 days in the control group, however, fibrous connective tissues invaded the upper area of the defects. So wound healing in control group is in the same manner as ordinal soft tissue healing because of granulation tissue invasion from the outside of the defect, which is the ordinal healing process of soft tissue. These findings support the previous suggestion by Matsuzaka et al\(^2\) that wound healing has not been completed at 10 days in the control group.

**Conclusion**

The findings of this study suggest that osteogenic cells differentiate into the osteoblasts in the membrane-covered bone defects and that the bone healing process will be completed at an early stage.

**Acknowledgments**

The authors would like to thank Ms Saori Takano for her technical assistance and Associate Professor Jeremy Williams for his assistance with the English of the manuscript. This research was supported by Oral Health Science Center Grant HRC7 from Tokyo Dental College and by a “High-Tech Research Center” project for private universities matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, 2006–2010, 2007–2010 (No 19592414) respectively.

**References**