Original Article

CHARACTERISTICS OF NEWLY FORMED BONE DURING GUIDED BONE REGENERATION: OBSERVATIONS BY IMMUNOHISTOCHEMISTRY AND CONFOCAL LASER SCANNING MICROSCOPY

KENICHI MATSUZAKA, MASAKI SHIMONO* and TAKASHI INOUE

*Department of Clinical Pathophysiology, Tokyo Dental College,
1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan*

** Department of Pathology, Tokyo Dental College,
1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan*

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Abstract

The purpose of this study was to investigate the characteristics of new bone formation during guided bone regeneration (GBR) using immunohistochemistry and confocal laser scanning microscopy. e-PTFE membranes were applied to defects created in the tibiae of rats, and some animals were sacrificed 6, 8, or 10 days later. Serial paraffin sections were cut, stained with H-E, and examined to analyze the ratio of new bone formation. Immunohistochemical staining with a monoclonal antibody specific for PCNA was used to evaluate the proliferating activity. In other experimental rats, calcein was injected at 6, 8, and 10 days after the surgery, and the animals were sacrificed 48hr after injection. Their tibiae were removed, and Villanueva bone staining was performed before observation using confocal laser scanning microscopy to investigate the mineralization of new bones. The bone occupation ratio increased day by day, but the experimental groups had significantly higher ratios than control groups (without membrane) at each of the time periods. However, PCNA positive cells decreased over time in all groups, and there were no significant differences among the groups. Mineralization occurred more rapidly in the experimental groups than in the control groups. These results suggest that GBR accelerates the migration of osteogenic cells, the formation of new bone, and mineralization in the defect created by the e-PTFE membrane.

Key words: GBR—Confocal laser scanning microscopy—Calcein—
Immunohistochemistry—PCNA

INTRODUCTION

Guided bone regeneration (GBR) is a method that facilitates repair of defective bones. The principle of GBR was originally based on guided tissue regeneration (GTR)

methods which isolated bone defects from gingival connective tissues^{1,6,8,9,13,21,24-27)} and created an exclusive space into which only cells from the surrounding bone could migrate. Becker *et al.*¹⁾ compared new bone formation with or without the GBR method. That study

showed that the GBR method achieved bone repair of defects in the same manner as bone grafts or substitutes. However, the characteristics of the bone and the cell behavior in this GBR method is still not clear.

Vital staining is a traditional technique using tetracycline and calcein to characterize bone mineralization^{2,11,12,30)}, recently, confocal laser scanning microscopy has also been used^{3,4,10,18)}. However, few studies have combined vital staining with confocal laser scanning microscopy⁴⁾. Although there are many reports examining of cell proliferation, differentiation or tissue regeneration^{7,14,15,19,23,28,32)}, there are only a few using immunohistochemistry. PCNA is a protein expressed by cells in the pre-mitotic stage, mainly from G1 to S⁵⁾. There are many reports examining PCNA positive cells in surgical pathology^{16,17,20,22)}.

The purpose of this study was to investigate the characteristics of bone tissues and the cell behavior in the defect underneath a barrier membrane using immunohistochemical techniques and confocal laser scanning microscopy.

MATERIALS AND METHODS

1. Surgical procedures and animals

Eighteen Sprague-Dawley rats were used in this study. Nine of those rats were used for immunohistochemistry, while the other nine were used for confocal laser scanning microscopy. Animals were anesthetized using sodium pentobarbital (Rabonal®), a skin incision was made along the lateral aspect of each leg, and then the muscles and periosteum were retracted to expose the lateral aspect of the tibia. Defects approximately 6 by 2.5 mm were prepared in the middle region of each tibia using a round burr mounted in a dental hand-piece cooled with phosphate-buffered saline (PBS). e-PTFE membranes 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 a control.

2. Light microscopy

Three rats were sacrificed with an overdose

Fig. 1 The scheme of the defect cross sectioned the tibia

CB: compact bone BM: bone marrow

of sodium pentobarbital at each day of 6, 8, and 10 after the surgery. Each tibia was removed and fixed in 4% paraformaldehyde for 2 days and then demineralized with 10% EDTA for 14 days before embedding in paraffin. Serial sections were cut, stained with hematoxylin-eosin, and then stained immunohistochemically with a monoclonal antibody specific for PCNA (PC10, DAKO, 1:100). Sections were de-paraffinized with xylene, rehydrated in 100% alcohol, and washed in distilled water. Endogenous peroxidase activity was blocked by incubating the sections with 3% H₂O₂ in methanol for 30 min. To prevent nonspecific binding, sections were then incubated in a 10% serum solution for 30 min in a 100% humidity chamber. PC10 was used at a dilution of 1:100 for 2 hr. Slides were washed in PBS and incubated with the secondary antibody for 30 min in a humidity chamber. After washing in PBS (5 min × 3 times), sections were stained with 3,3'-diaminobenzidine for 5 min, washed in distilled water, counterstained in hematoxylin, and coverslipped.

3. Morphometric analysis

Defects were divided into several parts in the

Fig. 2 HE staining of the experimental group in the central area at 8 days

The e-PTFE membrane keeps the outer tissues off. New bone is formed near the membrane (M: e-PTFE membrane).

Fig. 3 HE staining of the control group at 8 days

The defect is filled by muscle and fibrous connective tissues from outside (arrow).

photographs; the bone areas were the areas adjacent to the compact bone edge and the central areas longitudinally, and the upper and lower areas laterally (Fig. 1).

1) Ratio of newly formed bone

The ratio of newly formed bone was calculated by NIH Image (version 1.62, Power PC 603, Macintosh).

2) Ratio of PCNA positive cells (PCNA score)

The nature of staining and amount of PCNA immunoreactivity was evaluated for each part.

PCNA score

$$= \text{number of positive cells per } 1 \text{ mm}^2$$

Results are expressed as the percentage of the PCNA positive cells $\pm$ SD. The Student's *t*-test was used to analyze the data for the new bone ratios and the PCNA scores.

4. Confocal laser scanning microscopy

Calcein (10 mg/kg, Wako) was injected into three rats at either 8, 10, or 12 days after the surgery. Tibiae were removed and fixed in 4% paraformaldehyde for 2 days at 4°C and then immersed in Villanueva bone staining solution (Maruto) for 4 days at room temperature. Specimens were dehydrated with a graded ethanol series before being embedded in methyl methacrylate (MMA, Maruto) resin. Cross-sections approximately 200 μ m in thickness were cut at a right angle to the long axis of the defect

using a saw microtome (SP-1600, Leica) cooled with distilled water, then the surfaces were polished. Specimens were observed using a confocal laser scanning microscope (LSM-GB200, Olympus).

RESULTS

1. New bone formation

1) Histological observations

In the experimental group, a fibrous capsulation was observed at the outer area of the membrane at 6 days, but new bone had formed close to the membrane at days 8 and 10 (Fig. 2).

In the control group, muscle and fibrous connective tissue had invaded into the upper part of the bone defect at all experimental times examined (Fig. 3).

2) Ratio of newly formed bone

In the bone areas: The averages in both the experimental and the control groups increased over time. The averages in the experimental groups were higher than those of the control groups; the difference was statistically significant at 6 days ($p < 0.05$) (Fig. 4).

In the central areas: The averages in both the experimental and the control groups increased up to 8 days. At that time and thereafter, there were no further increases. The

Fig. 4 Ratios of newly formed bone and PCNA positive cells in the bone side

Fig. 5 Ratios of newly formed bone and PCNA positive cells in the central side

ratios in the experimental groups at 6 days were larger than those in the control groups, and the difference was statistically significant ($p < 0.05$) (Fig. 5).

Comparison of the bone areas and the central areas: The ratios of newly formed bone in the experimental and control groups were not statistically different in the bone areas or in the central areas.

Upper areas: The averages in both the experimental and the control groups increased over time. The ratios in the experimental groups were larger than those in the control groups, and were statistically different at 6 days ($p < 0.01$), and at 8 and 10 days ($p < 0.05$) (Fig. 6).

Lower areas: The averages increased until 8

days in both the experimental and the control groups. The ratios in the experimental groups were larger than that in the control groups, and the difference was statistically significant at 6 days ($p < 0.05$) (Fig. 7).

Comparison of the upper areas and lower areas: The ratio of newly formed bone in the lower areas of the experimental groups was significantly larger than in the upper areas at 6 days. The ratio of new bone in the lower areas in the control groups was significantly larger than in the upper areas at all times examined.

2. Confocal laser scanning microscopy

Newly formed bone had incorporated calcein and was observed as green or yellow green in

Fig. 6 Ratios of newly formed bone and PCNA positive cells in the upper side

Fig. 7 Ratios of newly formed bone and PCNA positive cells in the lower side

the confocal microscope. Fibrous connective tissues were stained red by Villanueva bone stain solution. Two labeling styles of mineralization by calcein were recognized. One type of labeling is a “diffusal type”, which stained all new bone material, indicating the early mineralizing phase. The other type of labeling is a “peripheral type”, which stained only the periphery, indicating the secondary mineralizing phase (Fig. 8).

In both groups at 6 days, calcein was detected diffusely on the new bone, and the experimental groups were stained more strongly than were the control groups (Fig. 9 a, b). In the experimental group at 8 and 10 days, calcein was detected only in the periphery of new bones (Fig. 9-c). In the control group at

8 days, calcein was detected in the periphery of new bone in the lower portion, but calcein stained diffusely in the bone and central areas of the upper portion. In the control group at 10 days, however, the pattern of new bone formation in the bone area of the upper portion and in both areas of lower portion was peripheral, whereas, in the central area of the upper portion staining, was still diffusal type (Fig. 9-d).

3. PCNA positive cells

1) Immunohistochemical observation

In the experimental group, several PCNA positive cells remained in the newly-formed bone tissues at 6 days (Fig. 10). At 8 and 10 days, there were no PCNA positive cells visible

Fig. 8 CLSM images of two styles of mineralization (arrow head)
a, diffusal type
b, peripheral type

Fig. 9 CLSM images of mineralization
a, The control group at 6 days after administration
b, The experimental group at 6 days after administration
c, The control group at 10 days after administration
d, The experimental group at 10 days after administration

Fig. 10 PCNA immunohistochemistry

- a, The experimental group at 6 days
Several PCNA positive cells are present in the newly formed bone (arrow).
- b, The control group at 6 days
PCNA-positive cells are found in the fibrous connective tissues.
- c, The experimental group at 10 days
PCNA-positive cells are not observed in the newly formed bone.
- d, The control group at 10 days
Several PCNA positive cells are detected in the newly formed bone (arrow).

in the bone, but cells in the fibrous connective tissues were PCNA positive.

In the control group, PCNA positive cells were found in the fibrous connective tissues at 6 days, and some PCNA positive cells were detected in the newly formed bones.

2) The ratio of PCNA positive cells (PCNA score)

In the bone areas: The averages in the control groups decreased in number until 8 days. The ratios in the experimental groups were higher than in the control groups, and were significantly different at 8 days ($p < 0.05$) (Fig. 4).

In the central areas: The averages in the experimental groups decreased over time, and the averages in the control groups decreased in number until 8 days. The ratios in the

experimental groups were higher than in the control groups, and were significantly different at 8 days ($p < 0.05$) (Fig. 5).

Comparison of the bone areas and central areas: The ratio of PCNA positive cells in the experimental and control groups were not significantly different between the bone areas and central areas.

Upper areas: The averages in the control groups decreased in number until 8 days. The ratios in the experimental groups were higher than in the control groups, and were significantly different at 8 days ($p < 0.05$) (Fig. 6)

Lower areas: The averages in the experimental groups decreased over time. The averages in the control groups decreased in number until 8 days. The ratios in the experimental groups were higher than in the control

Fig. 11 The scheme shows the characteristic of cellular activity and bone formation with or without GBR method

groups and were significantly different at 8 days ($p < 0.05$) (Fig. 7)

Comparison of the upper areas and lower areas: The ratio of PCNA positive cells in the experimental and control groups were not significantly different between the upper and the lower areas.

DISCUSSION

Wound healing of bone is started with the formation of a blood clot, and then the transmigration of fibroblasts and capillaries invade it from Haversian canals and Volkman canals. A few days later, fibroblasts proliferate and differentiate into osteoblasts, which form fibrous bone^{14,15,23,28,32}. The mechanism of bone formation in the space created by the barrier membrane progresses in the same manner, but the cell origin is still unclear. The ratios of newly formed bone in the experimental groups were

higher than in the control groups at all time periods examined and in all parts of the bone defects. These results suggest that only cells which form bones can proliferate in the space created by the e-PTFE membrane.

Vital staining is useful to characterize the development, remodeling and regeneration of bones, and experimental studies that have used fluorescent substances are well recognized^{30,31}. This method makes it possible to detect the mineralization of newly formed bones^{2,3,11,12,29,30}. Recently, many studies of tissue mineralization using confocal laser scanning microscopy have been reported^{4,10,18}. Kazama *et al.*¹⁸ suggested that sections used for light microscopic observations should be less than $10\mu\text{m}$ in thickness, and processing the thin ground sections of non-demineralized tissues is technically difficult^{4,18}. However, confocal laser scanning microscopy makes it relatively easy to observe the positional relations using thin sections and observing the sectioning

plane 1 μm in thickness. In our study, newly formed bones were labeled in 2 distinct manners by calcein. One type was a diffuse staining, and the other type was staining only in the periphery of new bones. It is known that the mineralization of bone begins with the secretion of matrix vesicles by osteoblasts, after which they are mineralized²⁹⁾. The diffusal type of calcein staining could be seen in the control groups at all time periods, but the peripheral type of staining was observed only at 8 and 10 days in the experimental groups. These findings suggest that the GBR method accelerates the mineralization of newly formed bone.

PCNA is a 36 kD acidic non-histone nuclear protein that functions as an auxiliary protein for DNA delta polymerase and is an absolute requirement for DNA synthesis⁵⁾. Its distribution in the cell cycle increases through the G1 phase, peaks at the G1/S interphase, and decreases through the G2 phase^{5,16,17,20,22)}. In this study, there was no significant difference between the control and experimental groups in terms of the ratio of PCNA positive cells. That ratio was decreased over time in both groups. When osteoblasts were enclosed in the bone matrix, the cells stopped proliferating and the new bone ratio increased. This must be the reason why the ratio of PCNA positive cells decreased over time. The ratios of newly formed bone in the experimental groups were higher in the upper areas than in the control groups, but the ratios of PCNA positive cells were not significantly different in those groups. This suggests that PCNA positive cells detected in the space created by the membrane are bone-forming cells, but in the upper area of the control group, they are almost all proliferations of other cells like fibroblasts. Although, a bone-forming cell like an osteoblast is suggested to be a specifically PCNA positive cell, the other PCNA positive cells like fibroblasts appeared as non-specific PCNA positive cells in this study.

Fig. 11 shows a scheme summarizing these results. The GBR method excludes the possibility of invasion by undesirable cells populating the wound area by means of the barrier membrane and by favoring the proliferation

of defined cells to obtain wound healing with newly formed bone. Furthermore, the newly formed bone generated with the GBR matures more rapidly than it does in the absence of the membrane.

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Reprint requests to:

Dr. Kenichi Matsuzaka
Department of Clinical Pathophysiology,
Tokyo Dental College,
1-2-2 Masago, Mihama-ku,
Chiba 261-8502, Japan