Statin-induced bone morphogenetic protein (BMP) 2 expression during bone regeneration: an immunohistochemical study

Statin induced bone morphogenetic protein (BMP)-2 expression during bone regeneration. An immunohistochemical study

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Abstract

Objectives: The purpose of this study was to investigate bone morphogenetic protein (BMP)-2 expression following implantation of a statin and recombinant human BMP-2 (rhBMP-2), and to compare the bone regeneration capability of these substances in the rabbit nasal bone using immunohistological methods**.**

Study design: Twelve adult male Japanese white rabbits (*n* = 12, age: 12–16 weeks, weight: 2.5–3.0 kg) were divided into three experimental groups and one control group. A total of 48 bone defects, four per rabbit, were created in the nasal bone while preserving the nasal membrane. In the experimental groups, one group was implanted with 10 mg statin dissolved in 0.2 ml water with an atelocollagen sponge (ACS), the second group was implanted with 5 μg rhBMP-2 with an ACS, and in the third group only the ACS was implanted. No material was implanted in the control group. Animals were killed at 1-, 2-, and 4- weeks postoperatively. The parts that had been operated on were removed and prepared for histological assessment. The expression of BMP-2 was evaluated using immunohistochemistry, and double-immunostaining for BMP-2 and Ki67 was observed by fluorescent microscopy.

Results: No significant differences were observed between the statin/ACS group and rhBMP-2/ACS group at 1, 2, and 4 weeks postoperatively. The number of cells which stained positively for BMP-2 increased significantly in both of the implanted groups when the control group ($P<0.0001$). The positive fluorescent double- immunostaining for BMP-2 and Ki 67 was similar in both implanted groups.

Conclusion: This study suggests that stain/ACS implants showed BMP-2 expression and osteoinductive activity that was similar to that of rhBMP-2/ACS implants.

Introduction

Autologous bone is the current gold standard graft material for the treatment of skeletal defects and fracture repair. However, the need for a second surgical site and the limited supply of bone available has led to the development of various alternative materials to autologous bone grafts.

Statins are specific, competitive inhibitors of 3-hydroxy-2-methyl-glutaryl coenzyme A (HMG CoA) reductase, and include naturally occurring lovastatin, chemically modified simvastatin and pravastatin[1-3] and the synthetically derived atorvastatin, fluvastatin, and cerivastatin. All of these agents are widely used to lower cholesterol, and they provide an important and effective approach towards the treatment of hyperlipidemia and arteriosclerosis[4]. However, statins also appear to modulate bone formation, inflammation, and angiogenesis. The suggestion that statins can increase bone formation has provided an exciting new direction for research as well as providing a greater understanding of the biological importance of cholesterol synthetic pathways. Simvastatin, mavastatin, and lovastatin have been shown to stimulate bone formation[5].

However, there are no reports on pravastatin inducing bone formation *in vivo*.

Bone morphogenetic proteins (BMPs) are active bone-inducing factors that act on immature mesenchymal cells, including osteoblasts, resulting in osteogenesis. So far, several types of BMPs have been isolated using molecular cloning, and recombinant BMP molecules have been synthesized[6]. Recombinant human BMP-2 (rhBMP-2) is a molecule that powerfully induces bone generation *in vivo*[7]. However, to optimize delivery at the bone repair site, rhBMP-2 requires a suitable carrier. Kusumoto et al.[8] reported that the atelopeptide type 1 collagen sponge is a useful carrier for osteoinduction by rhBMP-2.

To date, no experiments have been reported that have investigated the expression of BMP-2 induced by pravastatin during bone regeneration. The purpose of this study was to evaluate the bone regeneration process histologically and immunohistochemically using pravastatin or rhBMP-2 with atelocollagen sponge (ACS) implant materials in rabbit nasal bones, and to compare the bone regenerative capability of these factors in an animal model.

Materials and Methods

The experimental protocol was approved by the Institutional Committee for Animal Care, Kanazawa University.

Experimental animals

Twelve male Japanese white rabbits (12-16 weeks, 2.5-3.0 kg) were used in this experiment. They were divided into four groups – three experimental and one control. Each group was divided into three observation periods at 1, 2, and 4 weeks after implant.

Surgical procedure

The whole procedure was performed under sterile conditions. First, the animals were anesthetized with sodium pentobarbital (25 mg/kg), injected into the lateral ear vein. Then the hair in the nasal bone was shaved. After that, 1.8 ml of 2% lidocaine containing 1:80,000 epinephrine was administered into the operating site. Both the nasal bone and nasoincisinal suture line were exposed via a perpendicular incision. With the use of a fissure bur, four nasal bone windows were outlined. A surgical defect (5mm in diameter)

was made with a fissure bur using continuous saline irrigation. Great care was taken to avoid injury to the preserving nasal membrane**.** Four bone defects —three experimental and one a control—were created in each animal (Fig 1). The bone defect of this study is defined as a critical sized defect. This size has been reported to prevent spontaneous healing during animal's lifetime.[9]

Implant materials

The implant materials were a statin (Mevalotin®, pravastatin sodium, Sankyo Co. Tokyo, Japan), ACS (Teruplug®, Terumo Co. Tokyo, Japan), and rhBMP-2 with ACS (Teruplug®, Terumo Co. Tokyo, Japan). Forty eight bone defects were created at the nasal bones of Japanese rabbits, while preserving the nasal membrane. In the experimental group, defects were implanted with 10mg statin (Mevalotin®, pravastatin sodium, Sankyo Co. Tokyo, Japan), dissolved with 0.2 ml water and implanted into approximately 60 mm^3 (diameter 5 mm, thickness 3mm) ACS (Teruplug®, Terumo Co. Tokyo, Japan). Rh-BMP-2 with approximately 60 mm^3 (diameter 5 mm, thickness 3mm) ACS and approximately 60 mm^3 (diameter 5 mm, thickness 3mm) ACS alone were implanted. The rhBMP-2 was provided by Yamanouchi (Astellas) Pharmaceutical Co, Ltd, Japan. The rhBMP-2 was suspended in a buffer (pH 4.5) consisting of 5 mM

L-glutamic acid, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80 (Difco Laboratories, Detroit, MI, USA). It was kept at a temperature of –80ºC until needed, at which time it was thawed at room temperature. The ACS was cut into approximately 60 mm^3 (diameter 5 mm, thickness 3mm) blocks. In the rhBMP-2 implants, 5μg rh-BMP-2 was absorbed with approximately 60 mm^3 (diameter 5 mm, thickness 3mm) of ACS. This mixture was lyophilized before being implanted into the animals along the nasal bone. No material was implanted into the control group.

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Immunohistochemistry examination

The rabbits were sacrificed at 1, 2 and 4 weeks postoperatively and the specimens were fixed with 10% buffered formaldehyde overnight at 4°C, and demineralized with 14% EDTA for 4 weeks. The specimens were dehydrated with a graded ethanol series, cleared with xylene and embedded in paraffin. Five-mm sections were cut and mounted on gelatin-coated glass slides. Firstly, the prepared sections were treated with hematoxyline and eosin (HE). Then they were treated successively with 0.3% tween 20 in PBS for one hour for cell permeabilization, and then with 0.3% hydrogen peroxide in methanol for 10 min to inhibit intrinsic peroxides activity. They were then incubated overnight at room temperature with antibody BMP2 at 1: 100

dilutions in PBS. After washing with PBS, the sites of the immunoreactions were visualized by incubating the sections successively with biotinylated anti rabbit Ig G antibody at 1: 200 dilutions for 1hr, horseradish peroxides –conjugated streptavidin (Dako) at 1: 300 dilutions for 1 hour, and 0.01% diaminobenzidine tetrahydrochloride in the presence of 0.02% hydrogen peroxide in 50 mM Tris-HCL, pH 7.5 for 10 min.. The sections counterstained with hematoxylin were observed under on Olympus BX 50 microscope (Olympus; Tokyo, Japan). The sections were then dehydrated in alcohol and mounted for light microscopy to count the number of positively stained active cells in the regeneration site. The observation area was determined by the center of the bone defect area (material implanted area) (Fig.2). The number of stained cells per 1000 cells in this area was counted by hand using high magnification photomicrograph (×100).

Fluorescent double- immunostaining for BMP-2 and Ki 67 was performed. The sections were incubated with the mixture of rabbit BMP-2 antibody (1:500 dilutions) and Ki-67 (1:250 dilutions) in PBS overnight at 4°c. After washing with PBS, the sections were incubated with a mixture of Alexa Fluor 594-labeled anti-rabbit IgG and Alexa Flour 488-labeled anti-mouse IgG for 1 hr. They were then mounted in glycerol and subjected to examination with a fluorescent microscope (Olympus BX50/BX-FLA) first, using

green emission for Alexa Fluor 594 and then red emission for Alexa Fluor 488.

Statistical Analysis

Data of all the implanted materials were statistically analyzed with Stat View 4.5 (ABACUS Concepts, Inc., Berkeley, CA, USA). Differences between groups were analyzed by non-paired comparison using Scheffe's F test. Time-dependent changes were examined by analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

Results

Healing progressed uneventfully in all the animals and no postoperative complications were noted during the 4 week observation period. After resting for 3-6 days postoperatively, the animals could move and leap without any notable pain or limitation.

Histological examination

No findings suggestive of inflammation were observed at 1 week in any specimen. At 2 weeks, the operated area was partially covered with fibrous tissue in the

statin/ACS and rhBMP-2/ACS groups. At 4 weeks, the operated area was not restored completely and residual ACS was still visible. However, osteoblasts and fibroblasts in the implanted groups were found under microscopic observation using hematoxylin staining. But there were no changes in all the periods of the control groups.

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Statin /ACS treated with BMP-2 antibody

At 1 week, there were many positive immunostained cells (Fig. 3A). At 2 weeks, these positive cells were slightly decreased (Fig. 3 B) whereas the positive stained cells markedly decreased and reached the minimal level at4 weeks (Fig. 3C)

RhBMP-2/ACS treated with BMP-2 antibody

The positive immunostaining of BMP-2 was similar in the statin implanted group. Many Positive immunostaining cells were found at 1 week (Fig. 4A). But the positive stained cells decreased gradually at 2 weeks (Fig 4 B) and were at the minimum at 4 weeks (Fig. 4C).

ACS alone with BMP-2 antibody and Control with BMP-2 antibody

Moreover there were very few positive stain cells in the ACS and control groups at early period (Figs 5A and B).

Furthermore, we performed fluorescent double-immunostaining to compare the localization of the BMP-2 positive cells and Ki-67, a differentiating cell marker in the 4 weeks of both implanted groups. The BMP-2 positive cells were partially overlapped with the Ki-67 marker especially in the statin implanted area (Fig6 A, B, C) and similarly showed positive immunoreactivity as in the rh-BMP-2 implanted group (Fig 7 A, B, C).

Statistical analysis

For BMP-2 antibody, these time-course changes showed statistically significant differences by ANOVA (between subjects; $F=122.287$, $df=2$, $P<0.0001$; within subjects; $F=29.237$, $df=6$, $P<0.0001$). In the group treated with BMP-2 antibody, there were no significant differences detected at any period between statin/ACS and rhBMP-2/ACS. Similarly, when ACS was compared with the control there were no significant differences detected at any period. However, when statin/ACS was compared with ACS and the control group, the cell numbers were found to be significantly increased in all periods ($P<0.0001$). Similarly, when rhBMP-2/ACS was compared with ACS alone and the control group, the cell numbers were found to be significantly increased at all

the periods ($P<0.0001$) (Fig. 8 and Table 1).

Discussion

In this study we evaluated the bone regeneration process using two different implant factors and we compared the bone regenerative capability of these factors histologically and immunohistochemically in an animal model. In this study, ACS of the same size were used as carriers in all groups except in the control group to make the same amount of space available for bone regeneration using the different implant factors.

HMG-CoA reductase is one of the rate-limiting enzymes within the mevalonate pathway, through which cholesterol is biosynthesized. This enzyme is effectively inhibited by statins causing a reduction in blood-cholesterol levels. Other products of the mevalonate pathway are also important for the prenylation of some kinds of small GTPases. Since prenylated small GTPases are important for both activating osteoclasts and inhibiting the synthesis of BMP-2, statins inhibit the prenylation of small GTPases and, as a result, they can increase bone mass systemically[10].

In this study a new formulation of pravastatin/ACS material was used. The materials were dispersed in a water solution, which made them suitable for injection and

the injection was applied in a critical bone defect to evaluate the process of bone regeneration. Mundy *et al.*[5] first reported that statins stimulated bone formation in rodents *in vivo* and that they increased the volume of new bone in cultures from mouse calvarium. The enhancement of bone formation by statins is associated with an increase in the expression of BMP-2 through the activation of the gene promoter.

Wong *et al.* [11] reported that a pro-drug statin (Zocor®) can be dissolved in water and can induce the expression of BMP in surrounding tissues directly, and they found that following implantation of simvastatin, new bone was observed at 5 days post-operatively. However, there have been no reports about the induction of bone formation by pravastatin in an *in vivo* model. In this study, there were no findings to suggest the induction of inflammation in any of the specimens at 1 week post-operatively. Osteoblasts and fibroblasts were clearly observed at 4 weeks post-operatively, but the formation of new bone was not clearly observed at our study, because of the short time period for observation.

Osteogenesis induced by osteoblastic cells, is characterized by sequential events involving cell proliferation, followed by the expression of markers of the osteoblast phenotype and the synthesis, deposition, and mineralization of a collagenous matrix[12, 13] Bone formation depends mainly on the number of osteoblastic cells rather

than the activity of the osteoblasts[14]. The recruitment of osteoblastic cells plays a crucial role in osteogenesis. This suggests that new bone formation might be seen over a longer post-operative time period that in the current study.

Within subperiosteal implantation sites, BMP induces new bone formation in heterotrophic, intramuscular and orthotropic sites *in vivo*[15]. It is thought that BMP induces the differentiation of pre-vascular mesenchymal connective tissue cells into bone and cartilage. Various preparations of BMP have been reported to initiate new bone formation when implanted in animals[7, 8, 16-18] but there have been no reports on the use of BMP in the nasal region. In a previous study, a mesh-like woven bone structure was predominantly formed around the implant at 8 weeks post-implantation which diminished gradually[16]. In the present study where BMP-2 was implanted, new bone was not observed clearly, but cells associated with connective tissues, including fibroblasts were observed.

BMPs may initiate all of the molecular mechanisms required for the induction of bone formation, including the orderly migration, proliferation, and differentiation of osteoprogenitors, and the differentiation of mesenchymal cells into functional osteoblasts. The formation of new bone by bone-inductive proteins depends on the

quantity of the BMPs available and the delivery system or carrier used. Several carriers with suitable characteristics have been developed [7, 8, 17-19]. An ideal carrier should not only be resorbed and nonimmunogenic, but it should also provide a three-dimensional structure as a scaffold for new bone formation. Polylactic acid/polyglycolic acid (PLGA) gelatin sponge (PGS) and collagen are considered to be suitable as carrier matrices for BMP and appear to facilitate the effects of BMP. A BMP carrier should be nontoxic, biodegradable and conveniently sterilizable[20]. ACS appears to be particularly promising as a biomaterial for the transport of BMP[17, 19, 21]. In the present study, most of the ACS could not be seen at 4 weeks post-implantation. Inoda *et al.*[18] reported that following implantation of rhBMP-2 with collagen in the rat cervical model, most of the collagen had disappeared 6 weeks after implantation, and that all of the atelopeptide type 1 collagen sponge had been reabsorbed and substituted by mature bone 9 weeks after implantation.

We have reported previously[16] that newly formed bone observed at 8 weeks after implantation of doses of 5μg rh-BMP-2. In other studies, it has been proved that lower doses than 5μg rh-BMP-2 could induce new bone[7, 22, 23]. It was considered that rabbits are significantly more sensitive to lower doses of rh-BMP-2, so that the dose of rh-BMP-2 used in this study was adequate and enough to induce new bone formation.

However, recently Herford and Boyne [24] have reported that the doses 1.5mg/ml rh-BMP-2which was clinically used to induce bone in patients. When clinical dose is considered, higher dose may be needed to obtain significant bone formation.

On the other hand, there was no report regarding local use of pravastatin, so that it could be difficult to find the optimal dose of pravastatin. Wang et al, [11] reported previously that the newly formed bone observed after implantation of the doses 10mg simvastatin in rabbit bone defect. This dose was equivalent as a tablet in the oral administration for human. In the present study we used the doses 10mg of pravastatin in rabbit bone defect. This dose was equivalent as a tablet in the oral administration for human. Therefore, it was considered that the dose of pravastatin used in this study was enough to show the effect in rabbit bone defect. Furthermore, we tried to perform a preliminary study. From the results, when 10mg of pravastatin was used, osteoblast cell was shown significantly. On the basis of this result, we decided that the use of 10 mg of pravastatin was enough to induce new bone formation. However, further study is necessary to examine the effectiveness of various doses ranges of administration in or both rh-BMP-2 and statin in human.

A variety of markers, including Ki67, proliferating cell nuclear antigen

(PCNA) and estrogen receptors are frequently used to study cellular proliferation. In this study, we used the cell proliferation marker Ki67 which is expressed by proliferating cells in the late G1, S, and G2/M phases of the cell cycle. The nuclear localization of Ki67 and its particular association with the cell cycle indicates its importance in the regulation of cell division. Therefore, Ki67 has become an important marker of proliferating cells. Our immunofluorescence studies demonstrated that BMP-2 was co-localized with Ki67 in most areas, and that similar levels of expression were observed for both the statin/ACS and rhBMP-2/ACS implanted sites.

From the results of the ANOVA analysis, the change in expression of BMP-2 over time showed significant differences when the two implanted groups were compared with the control group ($P < 0.0001$). Furthermore, when the data of the multiple comparison analysis with Scheffe's F test at each period were added, our statistical analysis suggested that BMP-2 was expressed more intensely in both groups at 1 week post-operatively than it was at the other post-operative periods. However, the sample number in each post-operative period was so small and the data so variable that the significant differences obtained with Scheffe's F test could not be considered to be accurate. Therefore, further examination using a larger sample number is required.

These immunohistochemical studies demonstrated that BMP-2 was strongly expressed in the connective tissue and periosteum following the implantation of the statin and rhBMP-2 in the rabbit nasal bone. In addition, BMP-2 expression was similar in the sites implanted with statin or rhBMP-2 at every week that tissues were examined postoperatively. We conclude that statin/ACS showed BMP-2 expression and osteoinductive activity that is similar to rhBMP-2/ACS.

Acknowledgements

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Legends:

FIGURE 1. Intra-operative finding. (A) Bone defect was made at the nasal region. (B Diagram of the superior view of rabbit skull. Showing the sites of 4 surgically –created bone defects (a) Statin, (b) r-hBMP2, (c) atelo-collagen sponge, and (d) Control-no material were implanted within the defects.

FIGURE 2. The frontal section of the nasal region with implanted materials. The yellow colored rectangle area indicates the area of observation. Arrows show the area of implanted material. B: bone, S: nasal septum, N: nasal cavity.

FIGURE 3. Photomicrographs of statin/atelo-sponge group. Stained with BMP-2 antibody at 1 week (A), 2 weeks (B) and 4 weeks (C.) (Immunohistochemical staining, original magnification × 100), P: positive stained cell.

FIGURE 4. Photomicrographs of rhBMP-2/atelo-collagen sponge group. Stained with BMP-2 antibody at 1 week (A), 2 weeks (B) and 4 weeks(C). (Immunohistochemical staining, original magnification × 100), P: positive stained cell.

FIGURE 5. Photomicrographs of the control and and atelo- collagen sponge group. Control Stained with BMP-2 antibody at 1 week(A) and atelo- collagen sponge stained with BMP-2 antibody at 1 week(B) (Immunohistochemical staining, original magnification ×100), P: positive cell area.

FIGURE 6. Photomicrographs of immunofluorescence (IMF) BMP-2 and Ki 67 expression in the statin implanted site. (A) BMP-2, (B) Ki67 (C) BMP-2 and Ki67 co-localized area. Ep: Epithelial tissue of the nasal membrane, B: Bone, N: Nasal cavity, CT: Connective tissue.

FIGURE7. Photomicrographs of immunofluorescence (IMF) BMP-2 and Ki 67 expression in the rh-BMP-2 implanted site. (A) BMP-2, (B) Ki67, (C) BMP-2 and Ki67 co-localized area. Ep: Epithelial tissue of the nasal membrane, B: Bone, N: Nasal Cavity, CT: Connective Tissue.

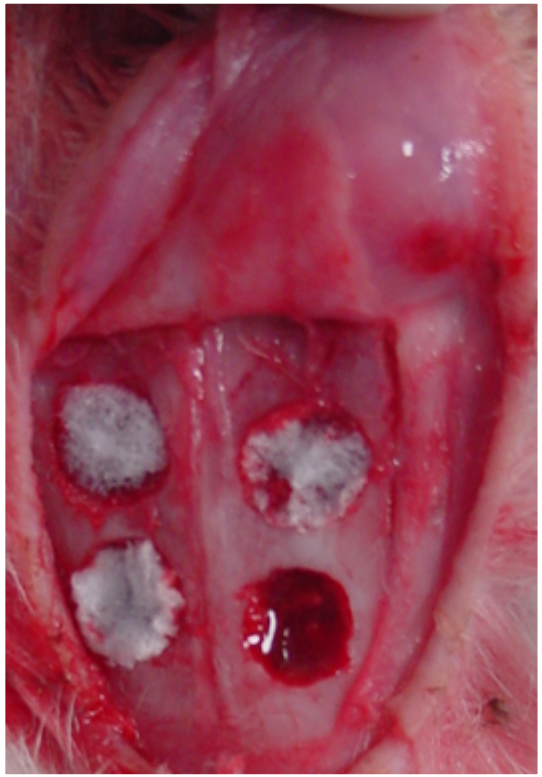
FIGURE 8. The ratio of BMP-2 positive cells. The time-course changes in all the groups showed significant differences with ANOVA.

Table 1. Results of the statistical analysis with Scheffe's F test. Expression of positive stained cells with BMP-2 antibody.

S: shows significant difference at $P<0.05$, and NS: shows no significant difference.

FIGURE 1

A



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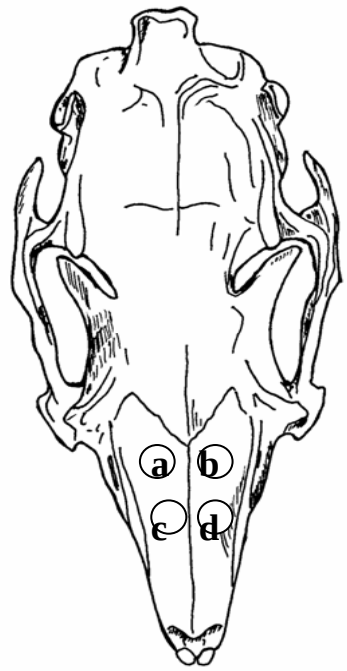


FIGURE 2

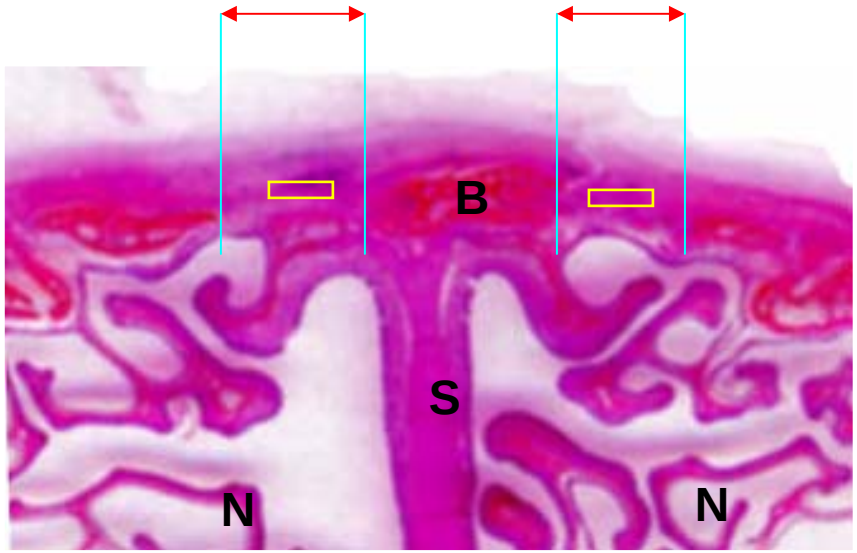
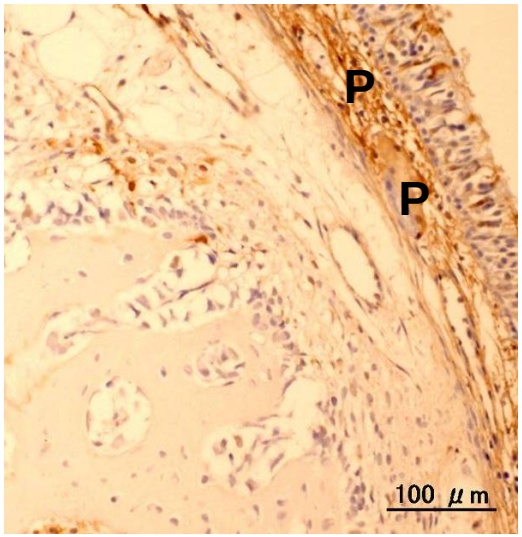
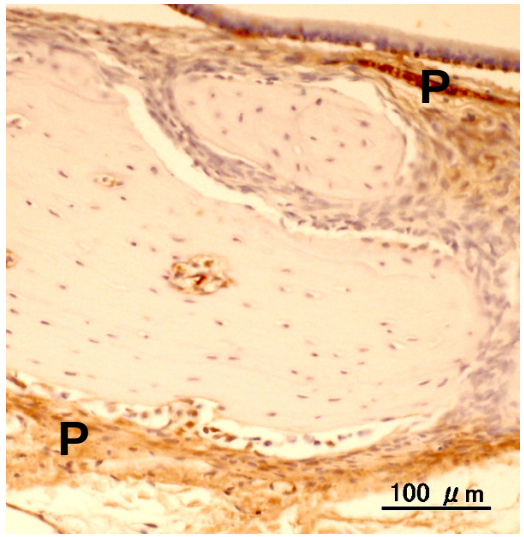


FIGURE 3



A



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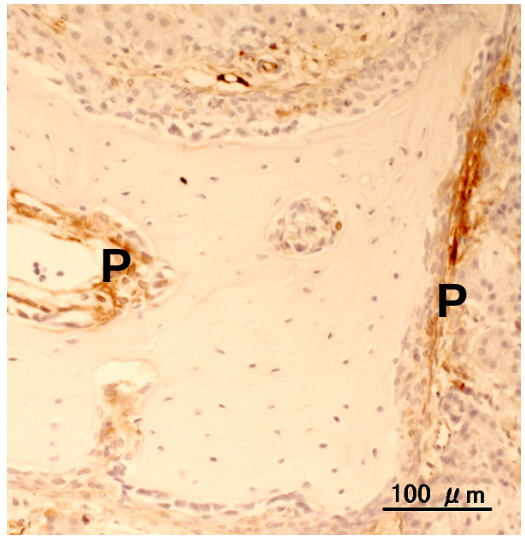
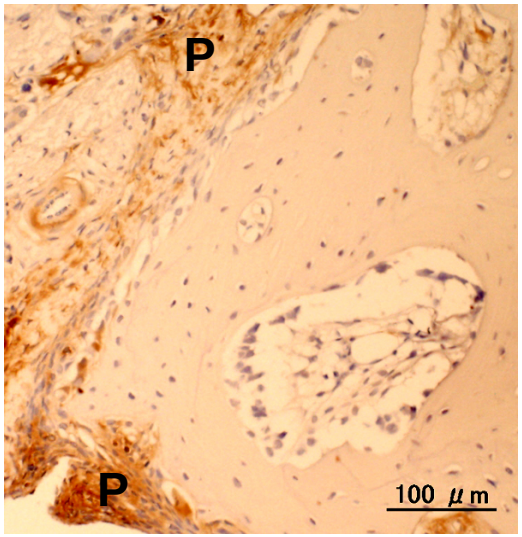


C

FIGURE 4



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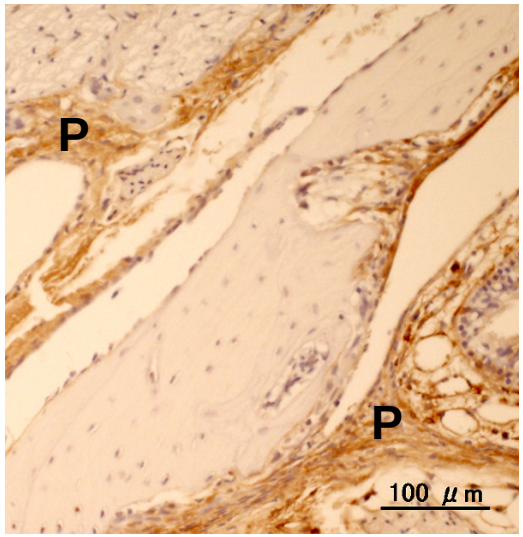
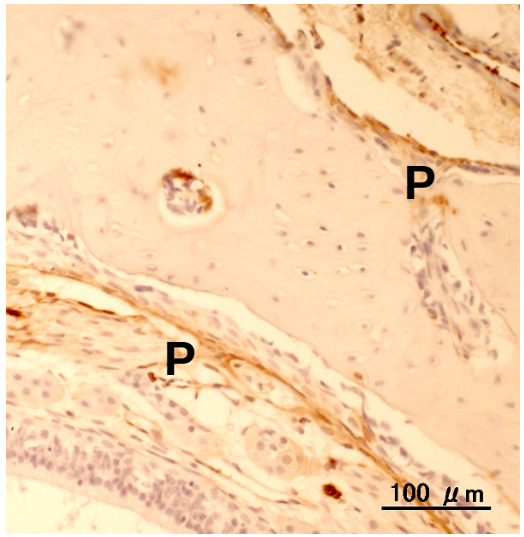
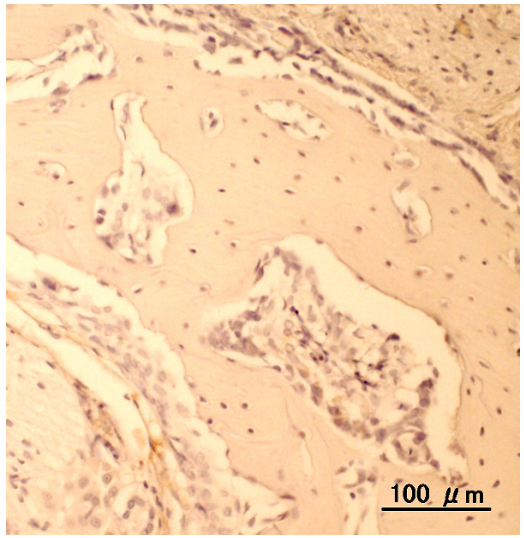


B



C

FIGURE 5



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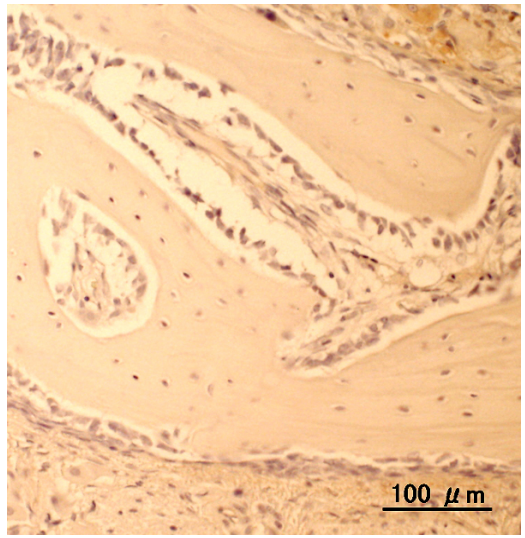
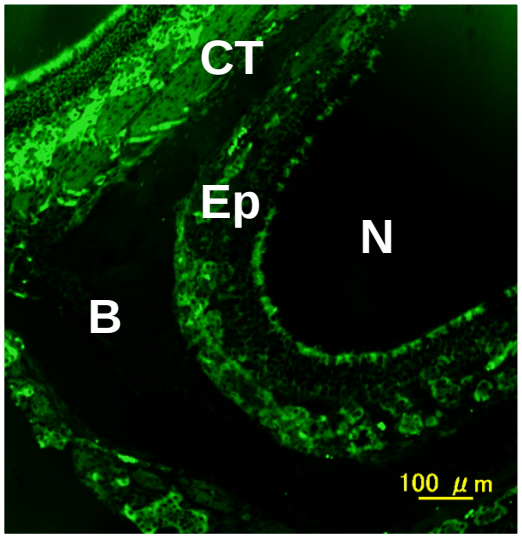


B

FIGURE 6



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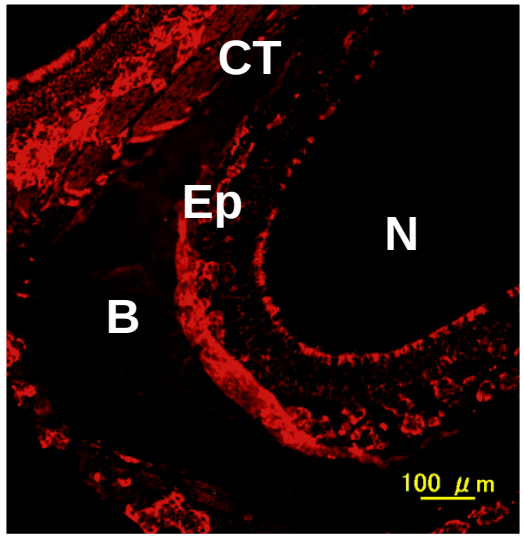

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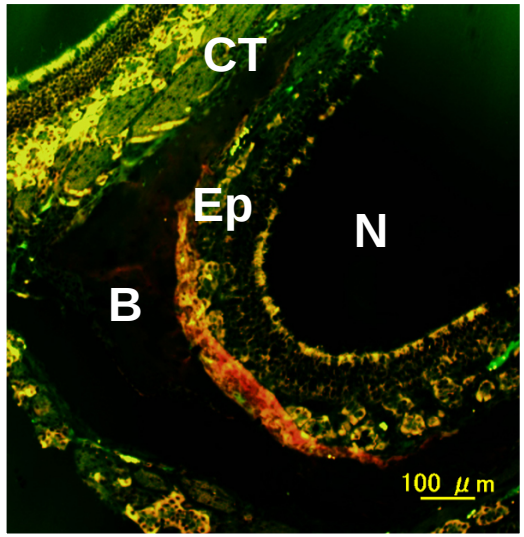
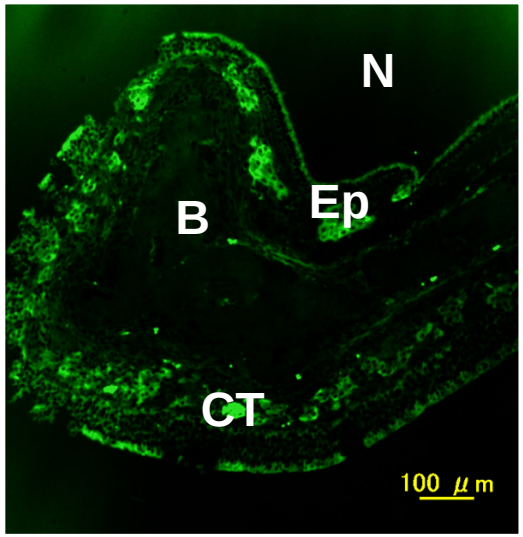


C

FIGURE 7



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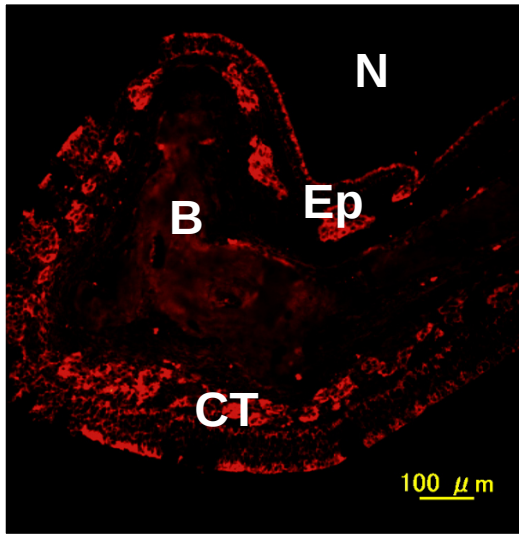

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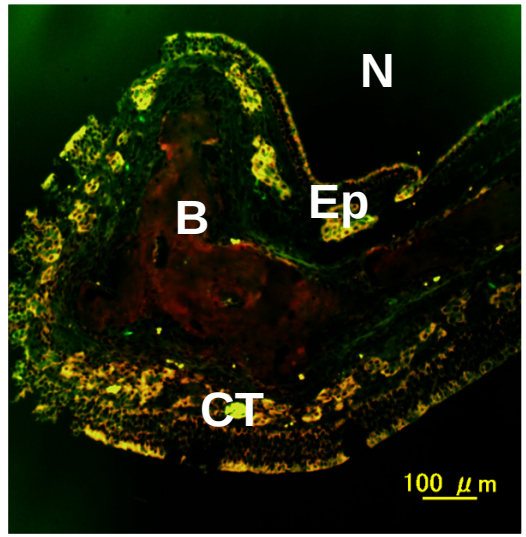

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FIGURE 8

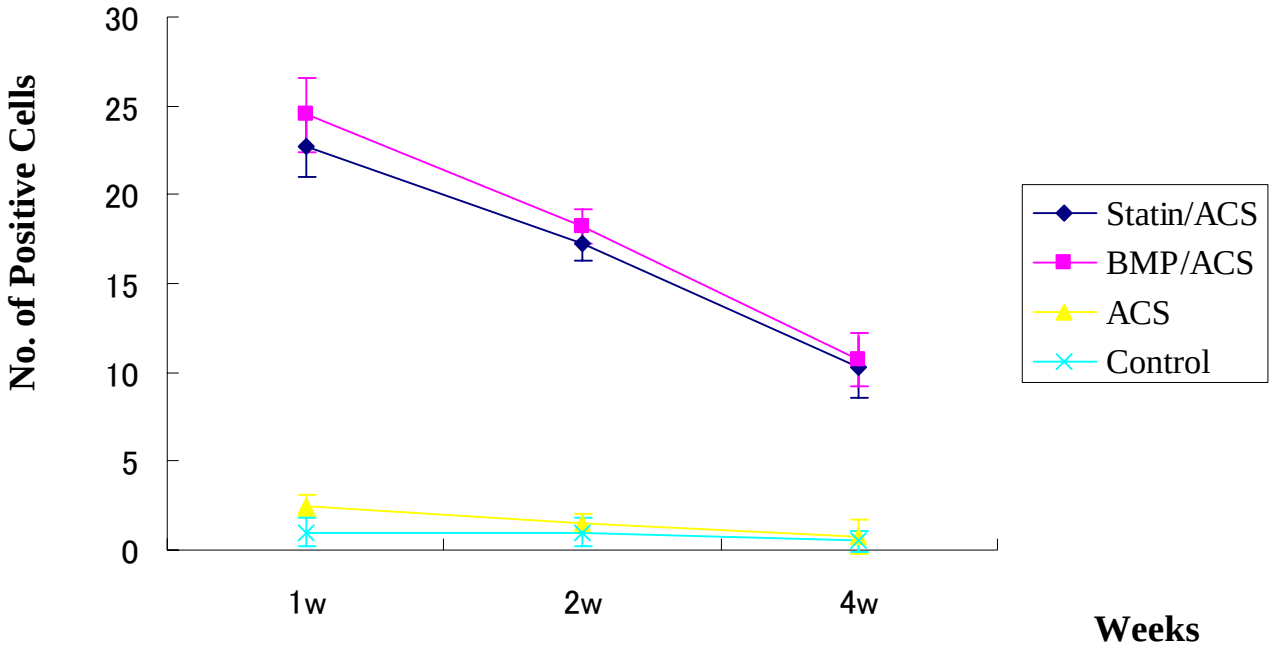
No. of Positive Cells
30
25
20
15
10
5
0
1w
2w
4w
Weeks
Statin/ACS
BMP/ACS
ACS
Control

FIGURE 9

BMP2 antibody			1week		2weeks		4weeks	
BMP2/ACS	vs	Control	<0.0001	S	<0.0001	S	<0.0001	S
BMP2/ACS	vs	ACS	<0.0001	S	<0.0001	S	<0.0001	S
BMP2/ACS	vs	Stain/ACS	0.430	NS	0.451	NS	0.956	NS
Control	vs	ACS	0.555	NS	0.870	NS	0.994	NS
Control	vs	Statin/ACS	<0.0001	S	<0.0001	S	<0.0001	S
ACS	vs	Stain/ACS	<0.0001	S	<0.0001	S	<0.0001	S