Cartilaginous repair of full-thickness articular cartilage defects is induced by the intermittent activation of PTH/PTHrP signaling

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Summary

Objective: We studied the effects of the transient activation of PTH/PTHrP signaling during the repair of 5-mm-diameter full-thickness defects of articular cartilage in the rabbit.

Materials and Methods: Cylindrical full-thickness articular cartilage defects of 5 mm in diameter were artificially created in the femoral trochlea of male adolescent Japanese white rabbits using a hand-drill. Recombinant human PTH(1-84) was then administered into the joint cavity continuously or intermittently for 2 weeks post-injury. The reparative tissues were histologically examined at 2, 4, and 8 weeks, and were also immunohistochemically examined for type II collagen. Double immunostaining analysis was also performed for the PTH/PTH-related peptide (PTHrP) receptor and proliferating cell nuclear antigen (PCNA) in the regenerating tissues.

Results: No evidence of cartilage formation was evident throughout the period of the experiments in injured animals administered saline alone. In contrast, cartilage formation occurred at 4 weeks in both the continuous and intermittent PTH-treated defects. At 8 weeks post-injury, for the intermittently treated defects, the regenerated cartilage successfully resurfaced the defects and the original bone-articular cartilage junction was recovered. In contrast, the defects were covered with fibrous or fibrocartilaginous tissues in the continuously administered group. PCNA and PTH/PTHrP receptor-double positive mesenchymal cells were significantly increased in both the continuous and intermittent PTH-treated defects at 2 weeks post-injury.

Conclusions: The present results suggest that the transient activation and release from PTH/PTHrP signaling during the early stages of the cartilage repair process facilitates the induction of regenerative chondrogenesis in full-thickness articular cartilage defects.

Key words: PTH/PTHrP signaling; chondrogenesis; tissue repair; articular cartilage; osteochondral defects.

Running title: Intermittent PTH treatment on chondrogenesis

Introduction

Cartilage is a typical avascular tissue and thus has a very limited capacity for regenerative repair. Full-thickness defects that penetrate articular cartilage and subchondral bone extending into marrow cavity undergo a repair process that eventually fills the defect cavities with fibrous, fibrocartilaginous, or hyaline cartilaginous tissue, depending on the location and size of injury[1, 2]. In a mature rabbit model, no chondrogenic repair response occurs for large cylindrical defects (≥ 5 mm in diameter) created in the femoral trochlea. In addition, only fibrous tissue has been shown to fill these defect cavities[3]. In contrast, smaller cylindrical defects (≤ 3 mm in diameter) are spontaneously repaired with regenerated hyaline cartilage[3]. Bone marrow derived chondroprogenitor cells have an essential role in the chondrogenic repair of the full-thickness defects of articular cartilage[2-4].

Various bioactive molecules, such as growth/differentiation factors and hormones, participate in the regulation of repair response in the defects[5]. Fibroblast growth factor-2 (FGF-2) also plays an important role in the induction of the chondrogenic repair response in cartilage defects by stimulating the mobilization and recruitment of proliferating chondroprogenitor cells into the defect cavities, resulting in the resurfacing the 5-mm-diameter defects via the regeneration of hyaline cartilage[4, 6, 7]. Hence, size limitations to the chondrogenic repair response in these types of cartilage defects can be alleviated by the addition of exogenous FGF-2[4]. When endogenous FGF-2 activity is neutralized by monoclonal antibodies, no chondrogenic repair response occurs even in the 3-mm-diameter defects, which are filled and resurfaced by fibrous tissue only[3].

The activation of parathyroid hormone (PTH)/PTH-related peptide (PTHrP)

signaling inhibits the differentiation of chondroprogenitor cells that are recruited to reparative tissue in cartilage defects[8]. The local administration of human PTH(1-84) completely disrupts the differentiation of chondroprogenitor cells in 3-mm-dimeter defects. There have been no adverse effects detected in the preceding steps for chondrogenesis upon exposure to PTH, i.e. the migration and proliferation of PTH/PTHrP receptor-positive chondroprogenitor cells. Chondrogenesis resumes when the defects are released from inhibitory PTH/PTHrP signaling during the early phase of repair at 2 weeks post-injury[9]. Mesenchymal cells that migrate to the site of repair have been found to be chondrogenesis-competent for only a limited time-span of around 2 weeks, but they lose their chondrogenic capacity after prolonged PTH treatment for 4 weeks. Most of the cells in the reparative tissue are PTH/PTHrP receptor-negative. Thus, 3-mm-diameter small defects continuously treated with PTH for 4 weeks are never covered with cartilage[9].

In our present study, we examined the effects of the transient activation and release from PTH/PTHrP signaling upon the repair response in 5-mm-diameter large full-thickness defects of articular cartilage in the rabbit. Our results indicate that the intermittent administration of PTH successfully induces the chondrogenic repair response in these defects and will be beneficial for the regenerative repair of large full-thickness defects of articular cartilage.

Materials and methods

Creation of full-thickness defects of articular cartilage in rabbits

Male adolescent Japanese white rabbits (n = 55), weighing 3-3.4 kg, were anaesthetized with sodium pentobarbital (30 mg/kg body weight intravenously). The right knee joint was then opened using a medial parapatellar approach under sterile conditions and the patella was dislocated laterally to expose the articular surface of the femoral trochlea. Full-thickness defects (5 mm in diameter; 4 mm in depth) were created in the weight-bearing area of the femoral trochlea with a hand-drill equipped with a 5-mm diameter drill-bit, as described previously[3, 8]. All animals were allowed to walk freely without a splint. Our research protocol was approved by the animal ethics committee of Kumamoto University School of Medicine, and animal care and experimental procedures were conducted in accordance with institutional guidelines (http://card.medic.kumamoto-u.ac.jp/card/japanese/kisoku/kisoku.html).

Administration of hPTH(1-84)

Recombinant human parathyroid hormone 1-84 (hPTH(1-84)) was expressed and purified from Escherichia coli by high-performance liquid chromatography using a cleavable fusion protein strategy as previously described[10]. Purified hPTH(1-84) prepared in this way was supplied by Chugai Pharmaceuticals (Tokyo, Japan) for the present study. The animals were fitted with an osmotic pump (Alzet, Model 2002, Alza Corp., Palo Alto, CA) connected to silastic medical grade tubing (0.75 mm inside diameter/1.45 mm outside diameter). A 5-mm-long piece of tubing was then introduced into the articular knee cavity through the articular capsule. An osmotic pump was then

placed subcutaneously into the hind region of the leg and the articular capsule and skin were closed independently with 4-0 nylon sutures[3, 8].

Fifty-five rabbits were given 5-mm-diameter defects and then divided into three experimental groups; one group was treated with PTH continuously for 2 weeks after injury (the continuous PTH group, n = 15), one group was treated with PTH intermittently for 2 weeks after injury (the intermittent PTH group, n = 15), and we analyzed an untreated control group (n = 20) which received saline alone for 2 weeks. In some experiments, additional untreated control rabbits (n = 5) which received saline for 1 week were sacrificed and subjected to histological analysis at 1 week post-injury. PTH was dissolved in sterile saline at a concentration of 50 μg/ml. The PTH solution was used in the continuous and intermittent PTH group. For animals in the continuous PTH group, the osmotic pumps were filled with 200 μl of sterile saline containing human recombinant PTH (50 μg/ml) and had a nominal pumping rate of 0.5 μl/h for 2 weeks. For animals in the intermittent PTH group, the osmotic pumps filled with sterile saline were connected to a length of tubing that had been precharged with the intermittent administration program. The program consisted of alternating segments of 5 μl PTH (50 μg/ml) solution and 5 μl sterile saline interrupted by 1 μl air bubbles. As saline from the pump gradually displaced the contents of the tubing, precharged PTH solution or sterile saline were alternatively discharged from the opposite end of the tubing. This pumping system results in the administration of the hormone solution for 10 hours with a 14-hour-interval. The animals were thus given an intermittent administration of PTH for two weeks post-injury. In all experimental group, the implanted osmotic pump and tubing were removed at 2 weeks after creation of the defects.

Histological and immunohistochemical analyses

Five animals from each study group were sacrificed at each time point with an overdose of sodium pentobarbital. The distal portion of each femur was then fixed in 4% paraformaldehyde at room temperature for 1 hour, decalcified with 10% EDTA for 3 weeks and then embedded in paraffin for subsequent histological analysis. To examine the sequential repair processes in each cavity, 5 μm transverse sections were cut in the transverse plane. The sequential repair process of each group was histologically analyzed as previously reported[8, 9]. For semi-quantitative analysis of the reparative tissue, the sections were examined in a blind manner by two observers, and were scored according to the modified Pineda scale (Table I)[11] and the O'Driscoll score (Table II)[12]. The modified Pineda scale used was inversely correlated to the original one so that a better recovery of the articular structure had a higher score. The score ranged from 0 (worst) to 14 (best). For quantification of subchondral bone repair, the cross-sectional area of the subchondral bone in the defects was measured. The section that had the maximum diameter (5 mm) was chosen as the representative section for each animal. The images were captured as digitized images with a scanner and stored on a personal computer. Both edges of the defect on the surface and a perpendicular (5 mm wide and 4 mm deep) rectangular region (original defect area) were delineated on the digital images, which were processed using Adobe Photoshop 3.0 to exclude cartilage, fibrous tissues and bone marrow. The cross-sectional area of bone within the original defect was determined using National Institutes of Health (NIH) image version 1.6 image analysis software (NIH, Bethesda, MD). The cross-sectional area of the subchondral bone was expressed as the mean and 95% confidence interval of five animals from each experimental group.

The presence of type II collagen in reparative tissues was examined immunohistochemically using a mouse monoclonal antibody against human type II collagen (Fuji Chemical, Takaoka, Japan) as this antibody cross-reacts with rabbit type II collagen[13]. The paraffin sections were deparaffinized and hydrated, and endogenous peroxidase activity was blocked by 0.5% hydrogen peroxide in methanol, followed by washing with 0.1% bovine serum albumin (BSA) in Tris-buffered saline (TBS). The sections were then treated with 500 U/ml testicular hyaluronidase (Sigma Chemical Co., St Louis, MO)[14]. Non-specific staining was reduced by incubation with horse serum. The sections were incubated with the primary antibody (dilution 1:100) overnight at 4°C, treated with the Vectastain avidin-biotin-peroxidase complex (ABC) kit (Vector Laboratories, Burlingame, CA), reacted with diaminobenzidine (DAB) solution, and then counterstained with hematoxylin.

The expression of PTH/PTHrP receptor protein in the reparative tissue was examined using a mouse monoclonal antibody raised against the opossum PTH receptor (Berkley antibody Co, Richmond, CA) using the same protocol described above[9]. The specificity and cross-reactivity of the monoclonal antibody to the rabbit PTH receptor were confirmed using the rabbit kidney and growth plate as a positive control [Fig. 5(E)], and the rabbit prostate [Fig. 5(F)] and thyroid gland [Fig. 5(G)] as a negative control[9]. The sections were counterstained with methyl green. The relationship between PTH/PTHrP receptor expression and cell proliferation was evaluated by double immunostaining for PTH/PTHrP receptor and proliferating cell nuclear antigen (PCNA). The paraffin sections were immunostained first for PTH receptor as described above, washed in 0.1% BSA in TBS and the sections were incubated with a monoclonal antibody against PCNA (dilution 1:100; Dakopatts, Copenhagen, Denmark) at room

temperature for 1 hour. They were next washed in 0.1% BSA in TBS and incubated with alkaline phosphatase (ALP) conjugated secondary antibody (dilution 1:100; Sigma, St. Louis, MO) for a further hour. The color development of the alkaline phosphatase reaction was carried out using ALP substrate kit I (Vector Red; Vector Laboratories, Burlingame, CA). A 1 mM solution of levamisole (Vector Laboratories) was added to block endogenous ALP activities. The sections were then counterstained with methyl green.

Quantitative analysis of the co-localization of PCNA and PTH/PTHrP receptor

To characterize reparative tissue immunohistochemically, we analyzed the cells in this tissue by dividing them into four sub-types: PTH/PTHrP receptor single-positive, PCNA single-positive, PTH/PTHrP receptor/PCNA double-positive and double-negative. Each sub-type was quantified according to the method of Aizawa and expressed as the percentage of the total number of cells in the defects[15, 16]. A section of each defect was then divided into 12 sampling square areas (1 mm × 1 mm) in which five fields were selected at a magnification of × 400. Because three sections from each paraffin bloc were used, the numbers of each of the four sub-types of spindle-shaped cells were determined in a total of 180 fields for each animal. The incidence of each cell type in the each case of reparative tissue was expressed as the mean and 95% confidence interval for five animals from each experimental group. Sections were examined in a blind manner.

Statistical analysis

Results were expressed as mean and 95% confidence intervals. Statistical

significance was estimated using Mann-Whitney's U test. A p value of < 0.05 was considered statistically significant.

Results

Histological features during the repair of articular cartilage defects

Large cylindrical full-thickness articular cartilage defects (5 mm in diameter) are not resurfaced with hyaline-like cartilage spontaneously, but filled with fibrous tissue only[1-3]. Control defects treated with saline alone were initially filled with blood clots but contained undifferentiated fibroblast-like cells within 2 weeks [Fig. 1(A)]. Although there was no sign of cartilaginous repair in these saline treated defect cavities [Fig. 2(B and C)], appositional repair of subchondral bone had already commenced by 2 weeks post-injury (Table III). At 8 weeks, the subchondral bone was almost completely reconstituted (Table III), but the defects were surfaced only by fibrous tissue [Fig. 1(G)]. No safranin-O staining was detected.

In defect cavities that had been continuously administered with 25 ng/h hPTH(1-84) for the initial 2 weeks post-injury [Fig. 1(B)] or had been administered intermittently with a-10-hours administration of 25 ng/h hPTH(1-84) followed by a 14-hour-interval during this same period [Fig. 1(C)], no apparent sign of chondrogenesis could be observed by histological appearance, similar to the saline-treated control defects [Figs. 2 and 3]. The subchondral bone area in either case of PTH administration at 2 weeks post-injury appeared to be smaller than that of the control defects, but similar to that of the control defects at 1 week after injury (Table III). This was not statistically significant however.

In the animal groups that had been continuously treated or intermittently treated with PTH for the initial 2 weeks, chondrogenesis was evident in the defect cavities by 4 weeks post-injury. In both groups also, the defects contained similar

amounts of safranin-O positive cartilaginous repair tissue on the subchondral bone [Fig. 2(E and F)]. Though the predominant tissue was fibrocartilage at the surface, the reparative tissue at the center of the defects contained chondrocytic cells of a rounded or polygonal shape [Figs. 2(B) and 3(B)] suggesting that short-term PTH treatments successfully stimulate chondrogenesis in the reparative tissue of 5-mm-diameter cartilage defects.

In the animal group treated with PTH continuously for the initial 2 weeks, the cartilaginous repair response could not be maintained at 8 weeks post-injury. Reduced safranin-O staining was seen in the reparative tissue covering the defects treated with continuous PTH at 8 weeks [Fig. 1(H)]. Moreover, most cells in the reparative tissue were poorly differentiated mesenchyme and there was little type II collagen immunoreactivity, with the exception of the deeper layer facing the regenerated subchondral bone surface [Fig. 4(D)]. Round-shaped chondrocytic cells were also only observed in the reparative tissue near to the subchondral bone. Surface lamination was further observed in these continuously treated defects at 8 weeks. The total modified Pineda score of the continuous PTH treatment group at 8 weeks was similar to that of the control group treated with saline alone ($P = 0.1745$), though significant improvements were seen in cell morphology ($P = 0.0203$) and matrix staining ($P = 0.0486$) [Fig. 2(A-C)]. The overall O'Driscoll score of the continuous PTH treatment group was also similar to that of the control group at 8 weeks ($P = 0.1337$) [Fig. 3(A)]. The score for nature of the predominant tissue [Fig. 3(B)], which includes cellular morphology and safranin-O staining of the matrix, was significantly higher ($P = 0.0486$) in the continuous PTH treatment group, compared to the control group at 8 weeks.

In contrast to continuous treatment, the defects intermittently treated with

PTH were successfully covered with hyaline-like cartilage by 8 weeks post-injury and the degree of safranin-O staining was comparable to that seen in the original articular cartilage [Fig. 2(I)]. As shown in Figure 4(F), type II immunoreactivity could also be seen abundantly throughout the resurfacing cartilage, which was smoothly connected with the original type II-collagen positive articular cartilage. The average modified Pineda score was also significantly higher than that of the control (total score: $P = 0.0153$, cell morphology: $P = 0.0062$, matrix staining: $P = 0.025$) [Fig. 2(A-C)]. Similar results were observed when the histological samples were scored using the O'Driscoll score (total score: $P = 0.0189$, nature of the predominant tissue: $P = 0.0104$) [Fig. 3(A, B)]. While the scores of the structural characteristics and the freedom from cellular changes of degeneration for the intermittent PTH treatment group were higher than those of control at 8 weeks, the differences were not statistically significant [Fig. 3(C, D)]. No significant difference in the degenerative changes of the adjacent cartilage was observed between any of the groups throughout the experimental period [Fig. 3(E)].

Chondrogenic responses in the defect cavities

In the control cartilage defects treated with saline alone, the expression of PCNA was only found in mesenchymal cells deeply located at the periphery of the defects (near to the subchondral bone surface) at 2 weeks post-injury. Most of the cells filling these saline-treated defects were PCNA-negative. The incidence of PCNA-positive cells was also below 20% on average in the control group (Table IV) and PTH/PTHrP receptor-positive cells were detectable only at the periphery of the defects, many of which were also PCNA-positive. In contrast, PTH/PTHrP receptor single-positive cells were widely evident in the center of the control defects, where only

a few cells were PCNA-positive [Fig. 5(B), right-hand side]. Hence, PCNA and PTH/PTHrP receptor double-positive cells were found almost exclusively at the periphery of the defects near the subchondral bone in the control group [Fig. 5(B), the left-hand side].

In contrast, PCNA-positive cells were frequently and almost uniformly detected in the PTH-treated defects, irrespective of whether the hormone was administered continuously or intermittently. Over 40% of cells in the reparative tissue were PCNA-positive in defects treated with PTH either continuously or intermittently at 2 weeks post-injury (Table IV), although no apparent difference in the cell density and the percent filling of the defects became evident in any of the experimental groups at the end of the PTH treatment at 2 weeks. It is noteworthy that most of undifferentiated cells in the PTH-treated defects were PTH/PTHrP receptor-positive. Numerous PCNA and PTH/PTHrP receptor double-positive cells are thus uniformly present throughout the reparative tissue in cartilage defects treated continuously or intermittently with PTH [Fig. 5(D)].

Discussion

Our present study showed that continuous and intermittent short-term PTH treatments in early stage of repair successfully induced chondrogenesis in the reparative tissue of 5-mm-diameter full-thickness articular cartilage defects. We also demonstrated that the defects treated with intermittent administration of PTH were repaired with hyaline-like cartilage by 8 weeks as similar to FGF-2-treated 5-mm-diameter full-thickness articular cartilage defects. However, when compared with FGF-2 treated defects, chondrogenesis in the PTH-treated defects was slightly delayed. In PTH-treated defects, there was no evidence of chondrogenesis at 2 weeks post-injury, whereas cartilage formation became evident as early as 2 weeks in FGF-2 treated 5-mm-diameter defects[3, 4].

The bone marrow contains stem cells that can differentiate into multiple mesenchymal lineages[17-19]. The recruitment of marrow-derived PTH/PTHrP receptor-positive chondroprogenitor cells and their subsequent proliferative expansion plays an important role in inducing the cartilaginous repair of full-thickness defects of articular cartilage, as previously reported[4, 20, 21]. In the rabbit model of these injuries, 5-mm-diameter large defects have been found to require supplementation of exogenous growth factors such as FGF-2 to evoke a chondrogenic repair response in defect cavities[3, 6, 7]. In addition, the accumulation of PCNA-positive cells in the PTH/PTHrP receptor-positive mesenchymal cell population in an early phase of the repair process was indicative of subsequent overt chondrogenesis at a later stage of repair[4, 9]. Hence, we analyzed the accumulation of proliferating chondroprogenitor cells by double immunostaining for the PCNA and PTH/PTHrP receptor in our current experiments. The present results of double immunostaining showed significant increase of PCNA and

PTH/PTHrP receptor-double positive mesenchymal cells in both continuous and intermittent PTH-treated defects at 2 weeks post-injury. These double positive cells were uniformly detected throughout the defects treated with continuous or intermittent PTH administration. These observations suggest that PTH treatment for an initial 2 weeks facilitates the recruitment of proliferating chondroprogenitor cells to the repair site.

It is known that PTHrP is secreted primarily by resting chondrocytes at the top of cartilaginous bone precursors and in the perichondrium of developing embryos, and acts on proliferating and prehypertrophic chondrocytes through the PTH/PTHrP receptor to slow the rate of chondrocyte differentiation[22-24]. However, recent animal studies have suggested that there are positive actions of PTH upon bone fracture healing and repair[25-27]. Using a rat fracture model, Nakazawa et al. have reported that daily subcutaneous injections of PTH stimulate chondrogenesis through the enhanced proliferation of mesenchymal cells[28]. Our present results are consistent with their contention that PTH increases proliferation of chondroprogenitor cells in the early stages of chondrogenesis, but not chondrocytes[28].

On the other hand, the sustained activation of PTH/PTHrP signaling clearly inhibits the differentiation of chondroprogenitor cells[8, 29]. The continuous local administration of PTH was found previously to completely inhibited cartilage formation, even in the 3-mm-diameter smaller defects and despite an apparent accumulation of proliferating chondroprogenitor cells in the defect cavities during the early stages of repair[8]. Accumulated chondroprogenitor cells remain chondrogenesis competent for at least 2 weeks in the defect cavities, but lose this ability by 4 weeks after the creation of cartilage defects[9]. We thus speculate from these earlier findings and our present

experimental setting that reparative tissue has to be released from PTH/PTHrP signaling so that accumulated proliferating chondroprogenitor cells can resume the process of differentiation into chondrocytes.

We observed cartilage formation in both of our PTH-treated animal groups at 4 weeks post-injury [Figs. 1, 2, and 3]. However, only the defects intermittently treated with PTH showed resurfacing by hyaline-like cartilage. The continuously PTH treated defects were covered with fibrous or fibrocartilaginous tissues at 8 weeks [Figs. 1 and 4]. The mechanisms underlying this differential repair outcome remain unknown. The molecular mechanism by which PTH promotes chondrogenesis in full-thickness defects of articular cartilage needs to be further explored in future studies.

The anabolic bone effects of PTH administration are well established. Intermittent PTH administration is also an approved treatment for enhancing bone mass in osteoporosis patients. Recent studies have strongly argued that the Wnt signaling pathway participates in the anabolic effects of PTH on bone formation and fracture repair[30-32]. However, neither the continuous nor intermittent administration of PTH in our current study produced a statistically significant difference in the time course of subchondral bone reconstitution (Table III). This suggests that the chondrogenic effects of PTH occur independently of bone formation. In summary, the short-term local administration of PTH shows therapeutic potential as part of a future strategy to regenerate articular cartilage.

Acknowledgments

Recombinant hPTH(1-84) was generously donated by Chugai Pharmaceuticals (Tokyo, Japan). This work was partly supported by the Grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Author contributions

Satoshi Kudo contributed to this work in the design of this study, acquisition of experimental data, analysis of data and drafting this manuscript as well as revision of this manuscript. Hiroshi Mizuta contributed in the design of this study, analysis and interpretation of data, and critically revising this manuscript. Katsumasa Takagi participated in this work in analysis and interpretation of data, and critical revision of this manuscript. Yuji Hiraki conceived and designed this study, and contributed this work in the analysis and interpretation of experimental data, and revision of this manuscript. All authors approved the submitted version of manuscript.

Conflict of interest

The authors have no conflict of interest to report.

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FIGURE LEGENDS

FIG. 1. The effects of the limited treatment of PTH on the repair of 5-mm-diameter articular cartilage defects in rabbits for the initial two weeks post-injury. These defects were treated with saline alone (A, D, and G), continuously with 25 ng/h hPTH(1-84) for (B, E, and H), or intermittently (10 hours per day with a 14-hours interval) with 25 ng/h hPTH(1-84) (C, F, and I) for the initial 2 weeks post-injury. The distal portion of each femur was cut in the transverse plane and the sections were stained with safranin-O at 2 weeks (A-C), 4 weeks (D-F), and 8 weeks (G-I) after the creation of the defects. Bar, 500 μm.

FIG. 2. Histological scores for the reparative 5-mm-diameter cartilage defects treated according to the modified Pineda scale (Table I). Values are the means and 95% confidence interval of the scores from the histological sections of five individual animals. In (A), total histological scores at 2, 4 and 8 weeks post-injury were compared. *Significant difference from control, $p < 0.05$. To evaluate cartilaginous repair in the induced defects, scores for cell morphology and matrix staining were performed as shown in (B) and (C), respectively. Continuous PTH treated defects showed higher scores compared to the control at 4 weeks (P = 0.0219) in the total score. Intermittent PTH treated defects showed higher scores compared to the control at 4 (P = 0.0219) and 8 weeks (P = 0.0153) in the total score. In the cell morphology, the continuous and the intermittent PTH treated defects showed higher score compared to the control at 8 weeks (control vs continuous PTH treated defect P = 0.0203, vs intermittent PTH treated defect P = 0.0062). In the matrix staining, the both PTH treated defects also

showed higher score compared to the control at 8 weeks (control vs continuous PTH treated defect P = 0.0486, vs intermittent PTH treated defect P = 0.0025).

FIG. 3. Histological scores for the reparative 5-mm-diameter cartilage defects according to the O'Driscoll score (Table II). Values are the means and 95% confidence interval of the scores from the histological sections of five individual animals. In (A), total histological scores at 2, 4 and 8 weeks post-injury were compared. *Significant difference from control, $p < 0.05$. To evaluate cartilaginous repair in the induced defects, scores for nature of the predominant tissue, which includes cellular morphology and safranin-O staining of the matrix, was performed as shown in (B). Continuous PTH treated defects showed higher scores compared to the control at 4 weeks (P = 0.0439) in the total score. Intermittent PTH treated defects showed higher scores compared to the control at 4 (P = 0.0472) and 8 weeks (P = 0.0189) in the total score. In the nature of the predominant tissue, the continuous and the intermittent PTH treated defects showed higher score compared to the control at 8 weeks (control vs continuous PTH treated defect P = 0.0132, vs intermittent PTH treated defect P = 0.0318). There was no significant difference among the group in the structural characteristics (C), the freedom from cellular changes of degeneration (D) at 8 weeks. During all of the experimental period, no significant difference in the freedom from degenerative changes in adjacent cartilage was observe between any of the groups (E).

FIG. 4. Immunohistochemical analysis of type II collagen at eight weeks post-injury in saline-treated control defects (A, B), defects treated with PTH continuously (C, D) or defects treated with PTH intermittently (E, F). Safranin-O (A, C, E) and type II collagen

immunostaining (B, D, F) were carried out. Arrows indicate the boundary between the original articular cartilage (right) and the reparative tissue (left). A positive immunoreactivity for type II collagen was observed throughout the reparative tissue in the defects intermittently treated with PTH. Bar, 1.25 mm for A, C, and E, 500 μm for B, D, and F.

FIG. 5. Double immunostaining for PCNA and PTH/PTHrP receptor at two weeks post-injury in the rabbit cartilage defect model, revealing mesenchymal cells in the upper left of the defects treated with saline alone (A, B) and treated with PTH intermittently (C, D). The transverse sections of femoral trochlea were stained with Safranin-O (A, C) or with PCNA and PTH/PTHrP receptor antibodies (B, D). PCNA was stained red with alkaline phosphatase substrate kit I, and PTH/PTHrP receptor was stained brown with immunoperoxidase-DAB. Panels B and D represent the PCNA or PTH/PTHrP receptor-positive cells in the corresponding areas denoted by asterisks in panels A and C, respectively. Bar, 1.25 mm for A and C, 50 μm for B and D. Panels E, F, and G show the single immunohistochemical staining of PTH/PTHrP receptor. The rabbit growth plate (E) was used as positive control and the rabbit prostate (F) and the rabbit thyroid gland (G) were used as negative control. Bar, 30 μm for E, 100 μm for F and G.

Figure 1

Fig. 1

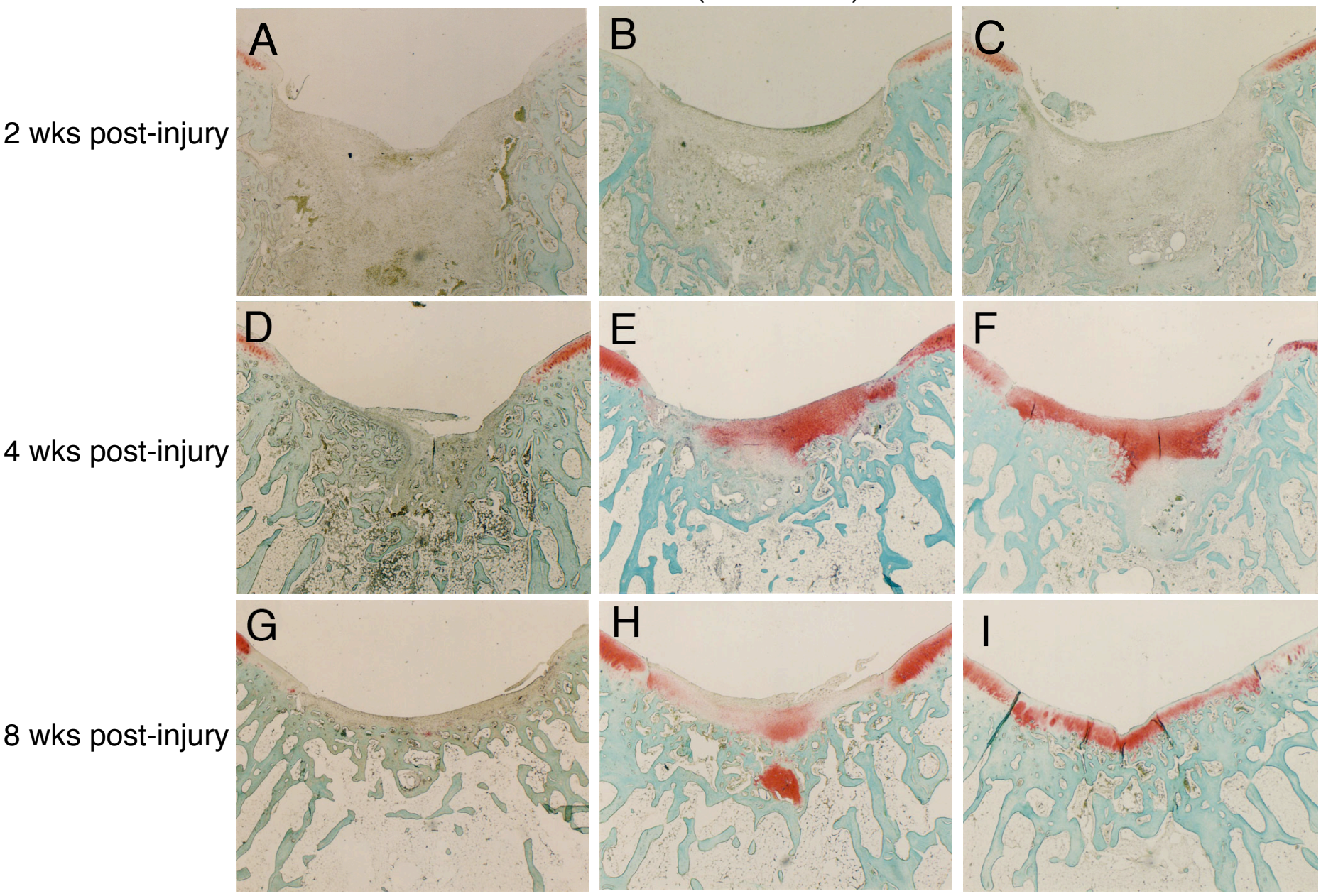

Figure 2

Fig. 2

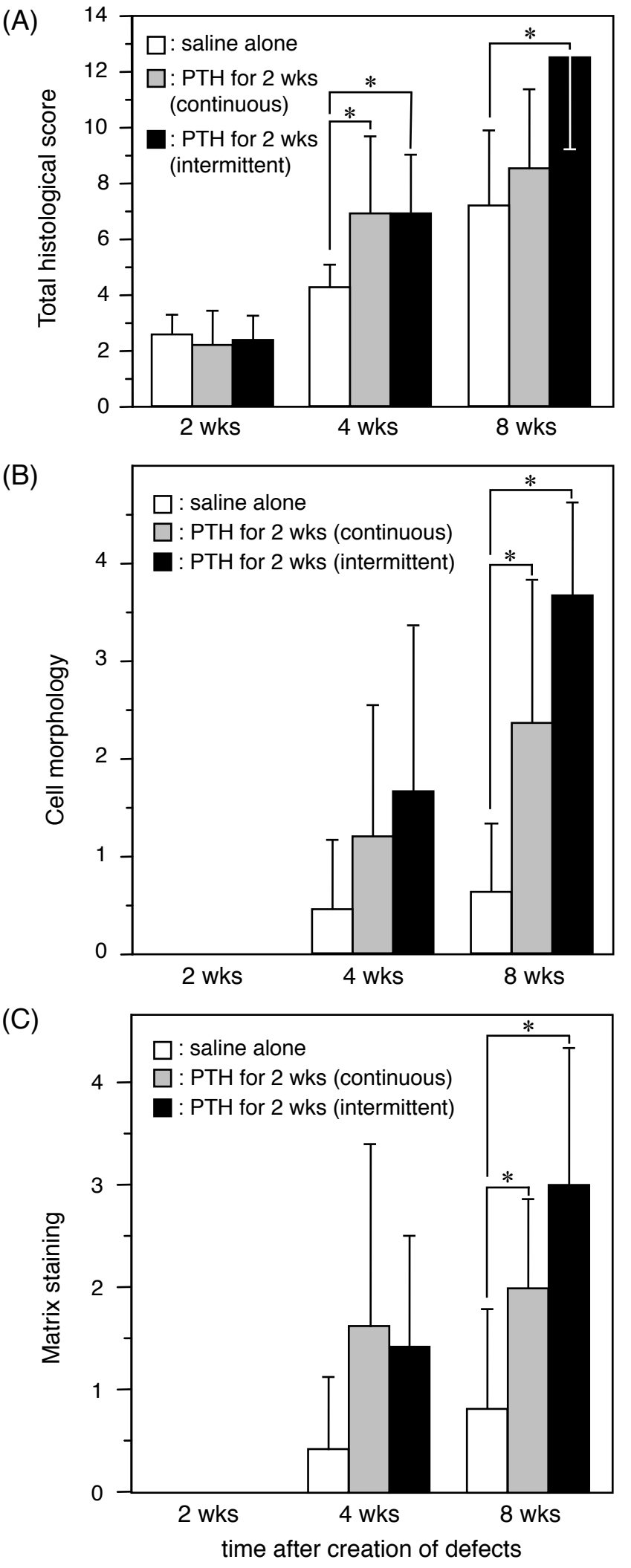

Figure 3

Fig. 3

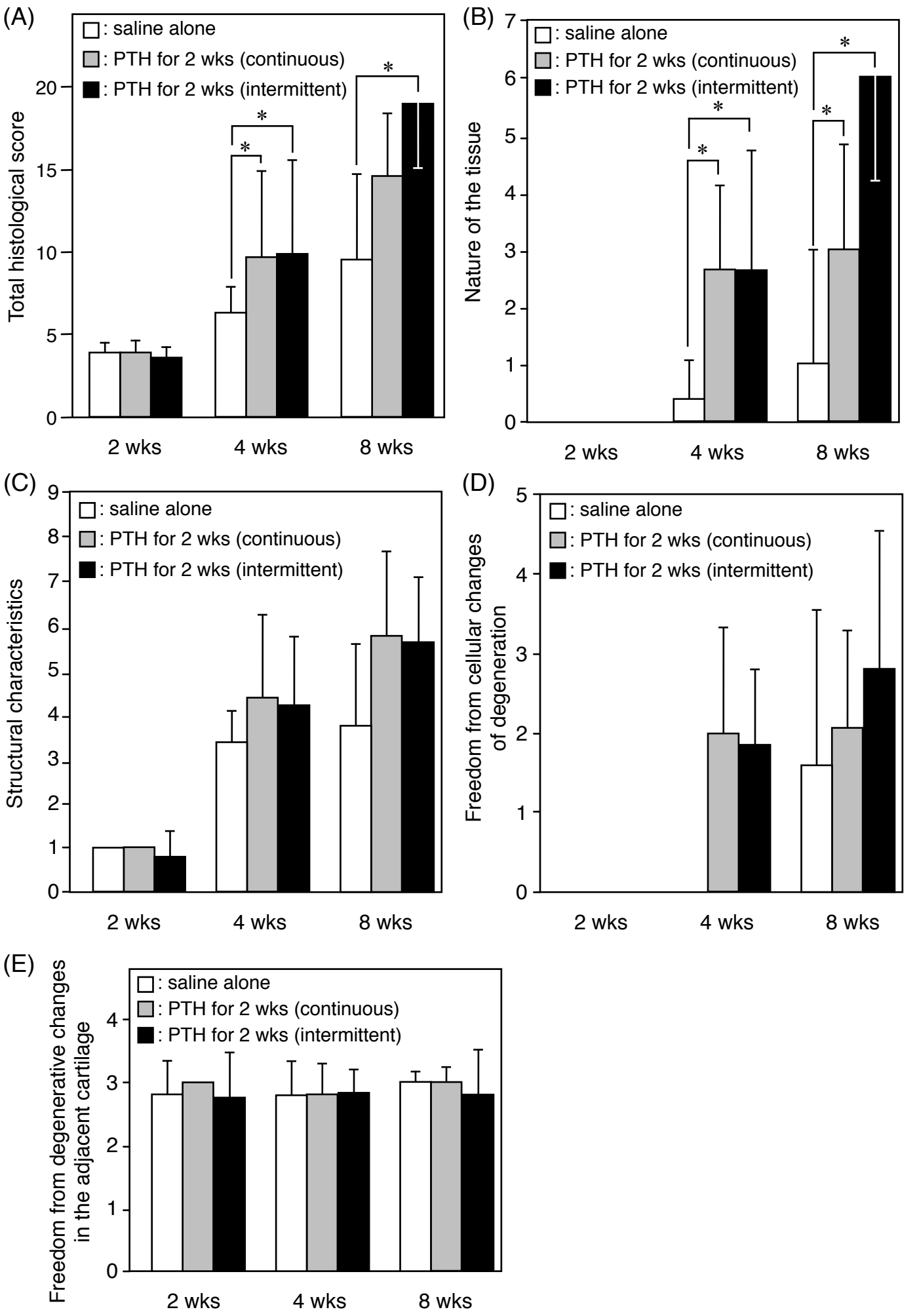

Figure 4

Fig. 4

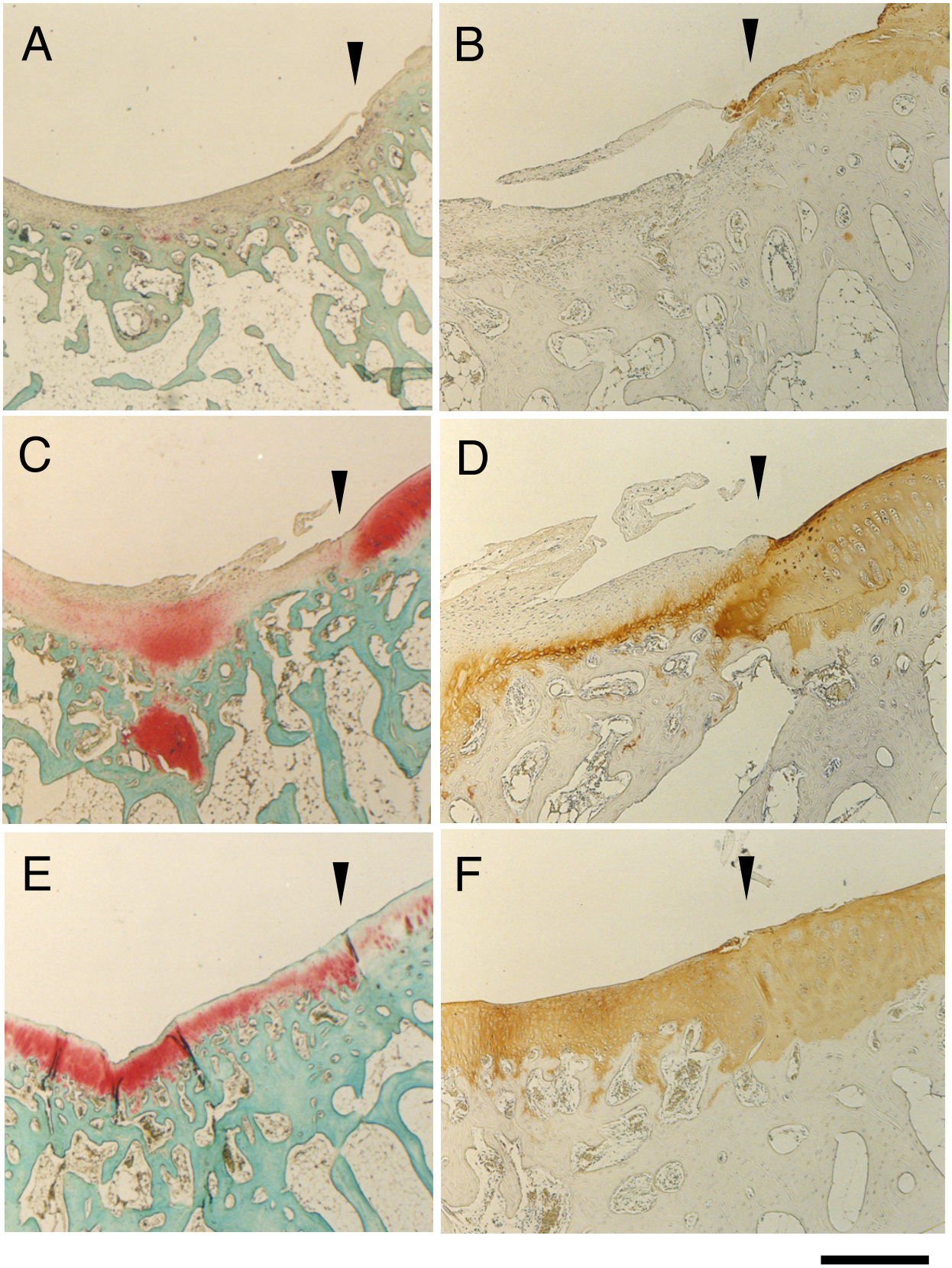

Figure 5

Fig. 5

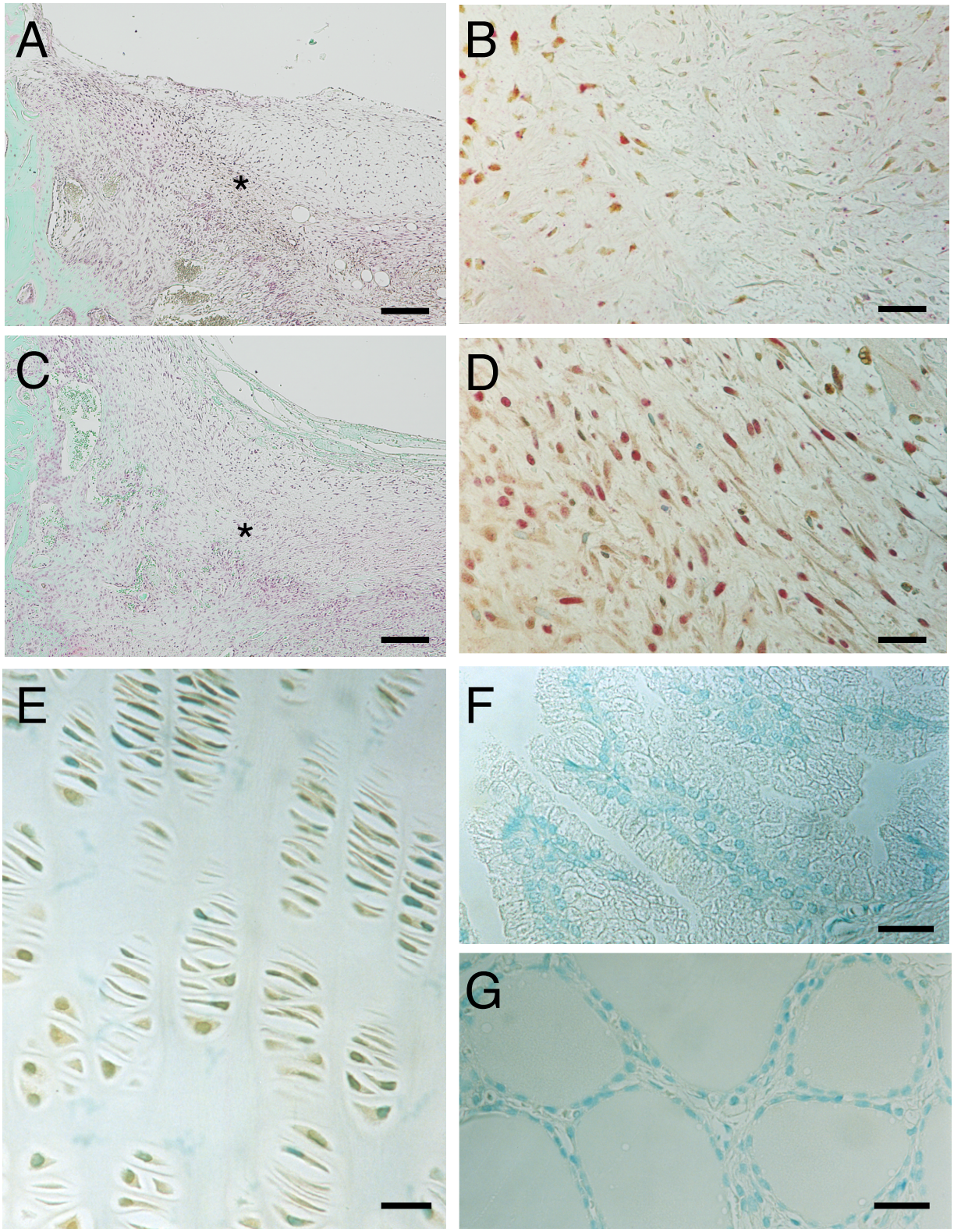

Table I

Table I. Scoring system for the histological appearance of full-thickness defects of articular cartilage*

Characteristics	Score
Filling of defects (%)	
125	3
100	4
75	3
50	2
25	1
0	0
Reconstitution of osteochondral junction	
Yes	2
Almost	1
Non close	0
Cell morphology	
Normal	4
Mostly hyaline and fibrocartilage	3
Mostly fibrocartilage	2
Some fibrocartilage, but mostly non-chondrocytic cells	1
Non-chondrocytic cell only	0
Matrix staining	
Normal	4
Reduced staining	3
Significant staining	2
Faint staining	1
No staining	0
Perfect score	14

*Modified from Pineda et al. (1992)[11]

Table II

Table II. Histological grading system for articular cartilage healing (the O'Driscoll score)*

	Score
Nature of the predominant tissue	
Cellular morphology	
Hyaline articular cartilage	4
Incompletely differentiated mesenchyme	2
Fibrous tissue or bone	0
Safranin-O staining of the matrix	
Normal or nearly normal	3
Moderate	2
Slight	1
None	0
Structural characteristics	
Surface regularity	
Intact	3
Superficial horizontal lamination	2
Fissures - 25 to 100 per cent of the thickness	1
Severe disruption including fibrillation	0
Structural integrity	
Normal	2
Slight disruption, including cysts	1
Severe disintegration	0
Thickness	
100 per cent of normal adjacent cartilage	2
50 - 100 per cent of normal cartilage	1
0 - 50 per cent of normal cartilage	0
Bonding to the adjacent cartilage	
Bonded at both ends of graft	2
Bonded at one end, or partially at both ends	1
Unbonded	0
Freedom from structural changes of degeneration	
Hypocellularity	
Normal cellularity	3
Slight hypocellularity	2
Moderate hypocellularity	1
Severe hypocellularity	0
Chondrocyte clustering	
No clusters	2
< 25 per cent of the cells	1
25 - 100 per cent of the cells	0
Freedom from degenerative changes in adjacent cartilage	
Normal cellularity, no clusters, normal staining	3
Normal cellularity, mild clusters, moderate staining	2
Mild or moderate hypocellularity, slight staining	1
Severe hypocellularity, poor or no staining	0

*Modified from O'Driscoll et al. (1988)[12]

Table III

Table III. Time course of subchondral bone area (mm^2) in the histological sections during repair of full-thickness articular cartilage defects

	1 wk	2 wks	4 wks	8 wks	24 wks
Saline alone	3.14 (2.63, 3.62)	4.02 (3.63, 4.41)	7.30 (6.66, 7.94)	8.88 (8.21, 9.54)	8.92 (7.26, 10.59)
hPTH(1-84)					
continuous		2.71 (1.52, 3.91)	6.03 (5.42, 6.64)	8.05 (6.88, 9.22)	
intermittent		3.05 (1.31, 4.79)	6.95 (5.39, 8.50)	9.35 (8.21, 10.46)	

Photomicrographs were taken of the histologic sections and scanned on a digital scanner. The computerized images were analyzed for cross-sectional area of subchondral bone in the defects using NIH image. Data are represented as a mean and 95% confidence interval of the mean (n = 5 for each group). There was no significant difference among the groups.

Table IV

Table IV. Cellularity and proliferation of the undifferentiated mesenchymal cells at two weeks after creation of defects

	Cell density (cells × 10^{-3} μm^2)	PCNA-positive cells (% of total)	Score of filling defects
Saline alone (n = 5)	2.9 (2.6, 3.1)	19.6 (13.6, 25.5)	2.8 (2.3, 3.4)
hPTH(1-84) for the initial 2 weeks			
continuous (n = 5)	2.8 (2.5, 3.2)	41.7 (21.2, 62.0) *	2.5 (1.9, 3.9)
intermittent (n = 5)	2.9 (2.3, 3.4)	43.8 (30.9, 56.7) *	2.6 (1.9, 3.1)

All nuclei were counted in a total of 96 fields in the 5-mm-diameter defects at 2 weeks using three section for each animal at a magnification of × 200, as described in Material and Methods. The PCNA-positive and -negative cells were counted in a total of 240 fields in the 5-mm-diameter defects at 2 weeks using three sections for each animal at a magnification of × 400. Data are represented as a mean and 95% confidence interval of the mean. Asterisks (*) signify a statistical significant difference in the incidence of PCNA-positive cells between the control (saline alone) and the continuous PTH treated defects (P = 0.0283) and between the control and the intermittent PTH treated defects (P = 0.009).