Local Injection of Thrombin-Related Peptide (TP508) in PPF/PLGA Microparticles–Enhanced Bone Formation during Distraction Osteogenesis

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ABSTRACT: We have previously demonstrated that injections of the thrombin-related peptide, TP508, into the lengthening gap have significantly enhanced bone consolidation in a rabbit model of distraction osteogenesis. This study was to further test the effect of a single TP508 injection in slow release preparation on bone formation during distraction osteogenesis. Rabbits had left tibiae lengthened unilateral lengthener at rate of 1.4 mm/day for 6 days. TP508 was injected into as the following: Group 1, TP508 in saline; Group 2, in PPF/PLGA [poly(propylene fumarate)/poly(D,L-lactic-*co*-glycolic acid)] microparticles; and Group 3, dextran gel only. All the animals were killed 2 weeks after lengthening. On radiographies, more bone was formed in the two TP508-treated groups at first and secnd week postlengthening than that of the control Group 3. Microcomputed tomography (microCT) at 2 weeks indicated that the most advanced bone formation and remodeling was seen in Group 2. The mean volumetric BMD of the regenerates was significantly higher in the TP508 treated groups compared to the control group ($p < 0.05$). Histological evaluations supported the radiographic and the microCT results. In conclusion, we have demonstrated that a single injection of small amount of TP508 (300 μg) at the end of lengthening phases has significantly enhanced bone consolidation process in a rabbit model of distraction osteogenesis. The delivery of TP508 in PPF/PLGA microparticles appears to lead to a better quality bone formation over the saline delivery, further examinations are needed to confirm if PPF/PLGA microparticles may be desirable drug delivery form in augmenting bone formation. © 2007 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 26:539–546, 2008

Keywords: thrombin-related peptide; TP508; poly(propylene fumarate)/poly(lactic-*co*-glycolic acid) (PPF/PLGA) microparticle; distraction osteogenesis; bone consolidation

INTRODUCTION

Thrombin is a mitogenic factor that presents in the early stage at the sites of tissue injury, involving fibrin clots and platelet activation. It is known that thrombin interacts with many cell types in both early and late stages of tissue repair/healing.[1–4] When clots dissolve, thrombin fragments activate specific receptors on many cells to initiate the healing process.[5,6] The thrombin-related peptide, TP508, is a synthetic 23 amino acid peptide, and represents a receptor-binding domain of the human enzyme prothrombin. TP508 mimics thrombin effects in accelerating initiation of wound healing by interacting with nonproteolytically activated receptors without affecting the blood clotting activity of thrombin.[4,6] TP508 has been shown to promote soft tissue, cartilage, and fracture repair.[7–11]

Induction of new bone formation through distraction osteogenesis (DO) techniques has widespread clinical applications in the treatment of

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bone defects, limb deformities, and fracture nonunions.[12–15] However, a long duration of bone consolidation phase is usually needed during DO treatments and augmentation of bone consolidation will be beneficial. Various growth factors, such as bone morphogenic protein (BMP)-2 and BMP-7, have been reported to promote bone consolidation during DO,[16,17] and we have recently demonstrated that repeated injections of TP508 (30–300 μg in saline) into the lengthening gaps have significantly enhanced bone consolidation in a rabbit model of DO.[18] With the continued improvement of drug delivery method, TP508 peptides have been incorporated into poly(propylene fumarate)-based (PPF) poly(lactic-*co*-glycolic acid) (PLGA) composite scaffolds to allow its slow release.[19,20] The aim of this current study was to exam if a single local injection of TP508 in the slow-releasing PPF/PLGA microparticles preparation will promote bone consolidation in DO.

MATERIALS AND METHODS

Animal Model of Distraction Osteogenesis and Experimental Groups

All animal experimental procedures were performed under the guidelines for Animals (Scientific Procedures) Act 1986, British Home Office. Midtibial osteotomies were performed in 22 adult male New Zealand White (NZW) rabbits (age, 24 weeks; mean body weight, 2.7 kg), with the tibiae stabilized with external fixators as previously described.[24,25] After a 7-day latency period, once daily lengthening was initiated at rate of a 1.4 mm/day for 6 days. Among the 22 experimental animals, 5 rabbits were euthanized before the experiment was completed, due to pinhole fracture ($n = 3$) and soft tissue complications ($n = 2$). These animals were excluded from the experimental groups. The remaining 17 rabbits were randomly divided into three experimental groups, each group consisting of at least 5 rabbits. In all groups, TP508 or control buffer were administered via percutaneous injection into the distraction (lengthening) gap under x-ray guidance at the end of the lengthening phase. Group 1 ($n = 6$) received injections of 300 μL saline containing 300 μg TP508; Group 2 ($n = 5$) received injections of 300 μL dextran gel mixed with PPF/PLGA microparticles containing 300 μg TP508; Group 3 ($n = 6$) received injections of 300 μL dextran gel alone. One hundred microliters was first injected into the center of the distraction gap and 100 μL each was then injected 0.3 cm distal and proximal to the central (first) injection point.

During the experiment period, animals were free to weight-bear on the operated leg with free access to food and water. All animals were euthanized at 2 weeks postlengthening. Immediately after sacrifice, the distraction regenerate plus 5 mm of the cortical bone proximal and distal to the regenerate was excised and fixed in 95% ethanol for further examination.

Preparation of PPF/PLGA Microparticles Containing TP508 Peptides

The detailed preparation techniques were described previously.[19,20] In brief, microparticles of 50:50 PLGA (Medisorb; Alkermes, Cincinnati, OH) loaded with TP508 (Orthologic Inc., Phoenix, AZ) were prepared by a double-emulsion, solvent extraction technique as previously described.[20] The final product was spherical microparticles with a mass loading of 0.066 g TP508/g microparticle as determined by an established solvent extraction technique. For PPF composite scaffold fabrication, PPF was mixed with PPF-DA (the double bond ratio of PPF:PPF-DA was 1:2) in the presence of CH_2Cl_2 and stirred overnight at room temperature to ensure proper mixing of the two viscous solutions. PLGA microparticles were located within the polymeric component of the scaffolds, microparticle mixtures (0.09 g microparticles/g polymer) were mixed in until homogeneously dispersed. All subsequent material handling occurred under sterile conditions. The complete scaffolds were exposed to an ultraviolet (UV) light for an additional 12 h after fabrication and stored at −20°C till use.

Radiographic Examination

Serial radiographs were taken at the day of surgery, end of lengthening, and 1 and 2 weeks postlengthening, using a high-resolution digital radiography system (Faxitron MX-20 with DC-2 option; Faxitron X-ray Corporation). The exposure condition was 32 KV, 10 ms at ×1 magnification. The percentage area of the distraction gap occupied by newly formed bone was scored by blinded observers, and it was graded (Table 1). The distraction gap was considered united when bony continuity was restored across >75% of its cross-sectional area. An average score from the two observers was taken for each set of radiographs. After decoding the animal groups, the means of the scores of each group were calculated and compared.

Peripheral Quantitative Computed Tomography (pQCT)

To assess the volumetric density of the regenerate, the excised bone specimens were scanned using a Stratec XCT 960M (Norland Medical Systems, Fort Atkinson, WI) with the software version 5.10 (Norland Stratec Medizintechinik GmbH, Birkenfeld, Germany). Briefly prior to scanning, calibration of the pQCT was routinely performed with a set of hydroxyapatite standards. The specimens were then placed in the holder and the centers of the regenerates were identified at the scout view window. Three slices were scanned, including the central slice, and one slice each 2 mm distal and proximal from the central slice. All slices were analyzed for total volumetric bone mineral density using the

Table 1. Mean Scores for the Final Radiographs

Group	Mean Scores	Number of Fully United Animals
1. TP 508 300 μg in saline	3.25*	4/6 (67%)
2. TP 508 300 μg in PPF/PLGA microparticles	3.71*	4/5 (80%)
3. Dextran only	2.60	2/6 (33%)

1. < 25% distraction gap filled with new bone.
2. 25% to 50% distraction gap filled with new bone.
3. 50% to 75% distraction gap filled with new bone.
4. > 75% distraction gap filled with new bone.
*$p < 0.05$, compared with Group 3.

manufacturer supplied software program, XMICE v1.3. A threshold of 1.300 attenuation units was selected, based on sampling of all scans, to include mineralized tissue and exclude soft tissue. A density threshold of 275 mg/cm^2 was used to differentiate bone from soft tissues. The mean volumetric bone mineral densities (BMD) of the regenerates from the three slices per sample were calculated and compared.

MicroCT Imaging

All the specimens were subject to MicroCT examinations using MicroCT-40 computed tomography system (Scanco Medical, Bassersdorf, Switzerland) before processing for histology. Details regarding the analysis software used in this study have been described previously.[21,22] Briefly, the unit consists of an x-ray source directed towards a cylindrical specimen holder. After traversing the sample, the x-rays are detected by a 1024×256 element CCD array that is piloted by a Compaq/HP Alpha Station operating in an open VMS environment. For scanning, samples were placed in a sample holder with the specimen's flat anterior surface facing downward. This sample orientation allows for longitudinal scanning of the single specimen. A control file, or measurement protocol, was created to define scanning parameters such as source energy, sample size, and image resolution. Parameters selected for this study included a source voltage of 55 kV and mA of 72. Sample measurements (scans) were performed on the samples using medium resolution settings. The sample measurement area selected for these scans consisted of the central-most region of the lengthening gap with a thickness of 2.00 mm. The medium-resolution scan yielded an image data set of two-dimensional (2D) longitudinal slices having an individual slice thickness/image resolution of 16 Am. For three-dimensional (3D) image analysis of the longitudinal image set acquired, a 4.0 mm^2 ROI was drawn centrally on slices composing a 2-mm sample region in the center of the distraction gap. On the medium-resolution scan, 60 consecutive slices were used for 3D reconstruction of the distraction gap.

Histological Examination

After the pQCT and microCT examinations, the samples were fixed in 10% buffered formalin for 48 hours and decalcified at 4°C over a period of 4 weeks in 14% EDTA in 0.1*M* Tris-HCl buffer, pH 7.2. All samples were then processed through graded alcohols, xylene, and embedded longitudinally (on their coronal plane) in paraffin wax. Seven-micrometer sections were cut at and stained with routine hematoxylin and eosin (HE) and Alcian blue/Sirius red. Alcian blue/Sirius red staining, following deparaffin, rehydration, nuclear staining with Weigert's hematoxylin, sections were stained with Alcian blue 8GX (0.1% in 1% acetic acid) and Sirius red F3B (1% in saturated picric acid). Alcian blue stains the proteoglycan-rich cartilage matrix (blue), while Sirius red stains the type I collagen fibril (red).

Statistical Analysis

The data from pQCT examination were analysed using a commercially available statistical program SPSS (version 11, Chicago, IL). Data from each group were tested by Mann–Whitney *U*-tests and differences were considered significant at $p < 0.05$.

RESULTS

General Observations

At the first week postlengthening, skin incision wounds in Groups 1 and 2 all healed with no sign of pin site infection and swelling. In contrast, at the end of 1 week postlengthening, the skin incision wound did not heal completely in 2 out 6 rabbits in Group 3; signs of swelling and tenderness at the operated legs were found in half of the animals in Group 3 and these animals did not appear to bear their weight on the operated legs. During the second week postlengthening, no apparent difference was noticed in animals in all groups.

Radiographic Evaluation of the Regenerates

The average (mean ± SD) lengthening of all the animals was 8.8 ± 0.6 mm. There was no difference found on the radiographs at the end of lengthening (Fig. 1). At the end of the first week postlengthening, radiographs showed that there was more bone

formation in the TP508-treated groups than in the dextran control group (Fig. 1). At second week postlengthening, Groups 1 and 2 had significantly greater mean x-ray scores compared to Group 3 (Table 1, $p < 0.05$, Mann–Whitney U-test). At 2 weeks postlengthening, the radiographic signs of cortical continuity were observed in 4/6 rabbits in Group 1, in 4/5 rabbits in Group 2, and in 2/3 of the Group 3 rabbits (Fig. 1 and Table 1). Radiolucent regions representing focal defects in the distraction regenerates were frequently seen in Group 3 (Fig. 1).

pQCT Results

As shown in Table 2, at 2 weeks postlengthening, the mean volumetric BMD of the regenerates were significantly higher in the TP508-treated groups when compared to the dextran control group ($p < 0.05$). There was no difference in the mean volumetric BMD between the two groups treated with TP508 (Table 2).

MicroCT Images

MicroCT images of the distraction gap tissues demonstrated advanced bone formation (there was no focal bone defects) and in the two TP508-treated groups (whereas focal bone defects were frequently seen; Fig. 2B, D) compared to the Dextran control group (Fig. 2F). The newly formed bone was much more evenly distributed across the entire distraction gap in Group 2 (Fig. 2D) compared to Group 1 (Fig. 2B). In Group 1, the newly formed bone in the distraction gap was less even and smooth comparing to the bone in Group 2. In Group 2, the callus was evenly formed across the gap with smooth shape of morphology, suggesting that bone remodeling was taking place (Fig. 2D); whereas in Group 3 larger focal defects were frequently seen and the new bone formation was poor in the medial-anterior side of the regenerate (Fig. 2F).

Histology Results

At 2 weeks postlengthening, the distraction gaps of the animals in Groups 1 and 2 mainly consisted of woven bone, formed in parallel to the distraction forces, with signs of callus remodeling (Fig. 3A, B). In Group 1, the distraction gaps had various degrees of consolidation, all animals had cartilaginous tissues in the regenerates (Fig. 3A). In Group 2, all the regenerates had shown advanced consolidation, and the callus was formed evenly in the distraction gap. There were small amount of cartilaginous tissues observed at the centers of the regenerates (Fig. 3B), but the amount was less than that of seen in Group 1. In Group 3 (dextran control), there were mixtures of fibrous, cartilaginous, and bony tissues in most of the regenerates. Even in the regenerates considered consolidated on x-ray, focal defects with fibrous and cartilaginous tissues were frequently seen (Fig. 3C). In comparison, bone consolidation and remodeling was most advanced in the Group 2, where 4/5 of the animals' distraction gaps were completely united with well-organized woven bone (Fig. 2B). There were numerous blood vessels in the distraction regenerates in Groups 1 and 2 (not shown). Focal cartilaginous tissues were still evident in some animals in Groups 1 and 2, more so in Group 1. Large amounts of fibrous and cartilaginous tissues were only visible in Group 3 at 2 weeks postlengthening. Samples from Groups 1 and 2 showed almost no sign of inflammation. Inflammatory cells (mainly lymphocytes) were only found in Group 3 (data not shown).

DISCUSSION

During distraction osteogenesis, bone formation occurs in parallel with the tension force, leading to rapid bone formation.[12,13] This technique has been widely used clinically for the treatment of many challenging orthopedic conditions, such as limb reconstruction following bone loss cased by trauma, infection, and tumor resection.[14,15] However, a lengthy period of external fixation is usually needed to allow the new bone to consolidate and complications such as refracture at the distraction gap are often seen.[14,15,23,24] Previously reports using rabbit model of DO have shown that the optimal rate of lengthening is 0.7 mm/day, twice daily lengthening[24,25]; when lengthened at a higher rate (>1.3 mm/day), the quality of bone formation is poor.[24–26] In this study, we deliberately chose a lengthening rate of 1.4 mm/once a day to mimic clinical situations of poor bone formation during DO, which has been used in our previous study[18] and the poor bone formation is comparable as a rapid lengthening rate at 2.7mm/day, twice a day lengthening, as previously reported.[24–26]

In previous study we have shown that the consolidation of the distraction regenerates was enhanced when TP508 (300 μg in saline) was injected twice into the distraction gap at the early stages of distraction osteogenesis.[18] In this study,

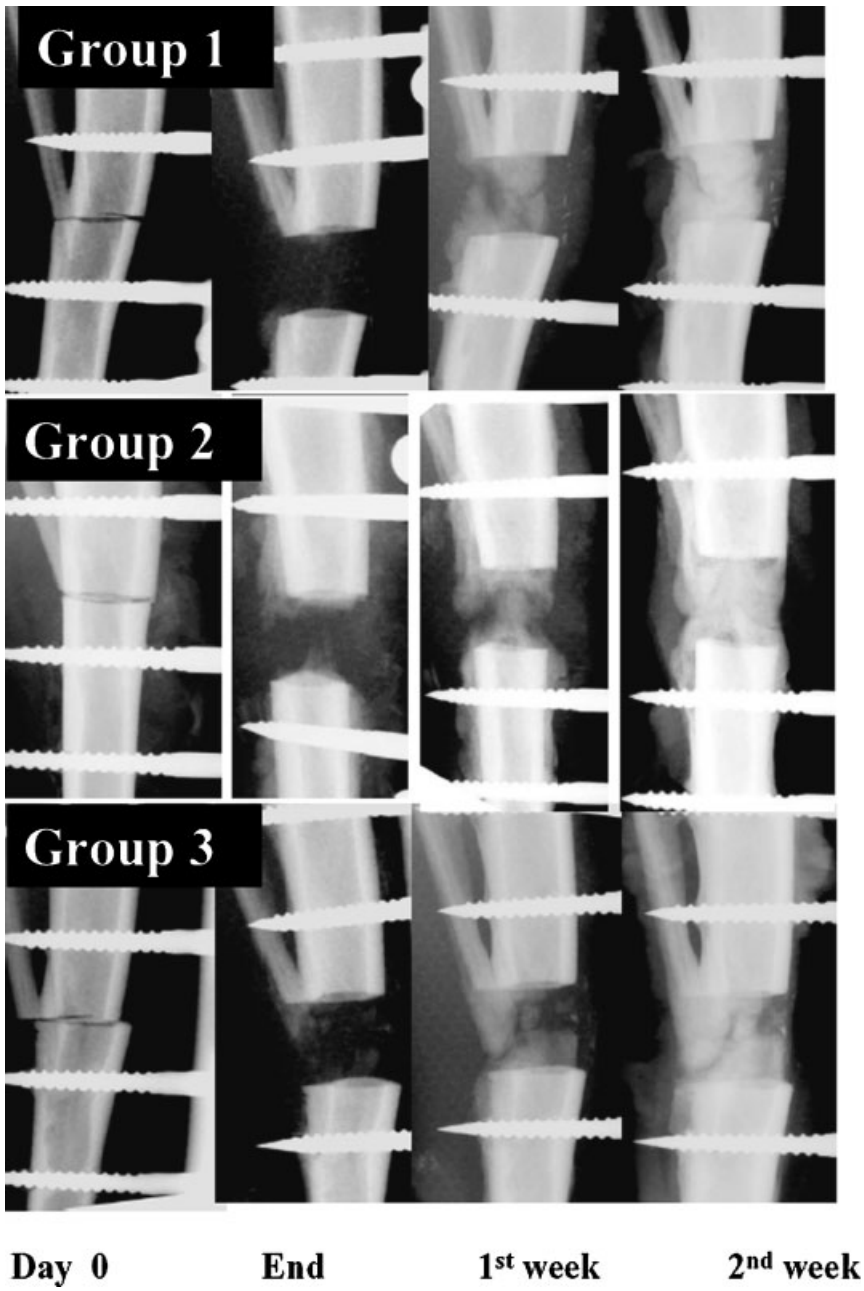

Figure 1. Representative radiographs of all experimental groups at day 0, end of lengthening, 1 and 2 weeks postlengthening. There was a significant increase in callus formation in the TP508-treated groups compared with the dextran control group at 1 and 2 weeks postlengthening. Most advanced bone formation and consolidation was seen in the group with TP508 300 μg treatment with PPF/PLGA microparticles (Group 1). At second week postlengthening, although bony unions were seen in all groups, radiolucent regions representing focal defects in the distraction regenerates were frequently seen in Group 3.

significant increases in bone mineral density of the distraction regenerates and improvement of the quality of bone formation were achieved either by a single local injection of TP508 in saline or PPF/PLGA microparticles in a rabbit model of DO. Although bone mineral density of the regenerates was not significantly different between the two TP508-treated groups (TP508 in saline vs. TP508 in PPF/PLGA microparticles) in this study, the group receiving TP508 in PPF/PLGA microparticles had higher mean bone mineral density (522.73 ± 41.07 mg/cm^2 vs. 495.48 ± 50.94 mg/cm^2) and demonstrated a superior histology of newly formed callus than that of TP508 injection with saline. The

Table 2. Mean Bone Mineral Density (BMD) of the Newly Formed Bone (mg/cm^2)

Group	BMD (Mean ± SD; mg/cm^2)	p Value
1. TP 508 300 μg in saline	495.48 ± 50.94	0.045 (vs. Group 3)*
2. TP 508 300 μg in microparticles	522.73 ± 41.07	0.026 (vs. Group 3)*
3. Dextran only	436.78 ± 36.77	0.55 (Group 1 vs. 2)

*$p < 0.05$, Mann–Whitney U-test.

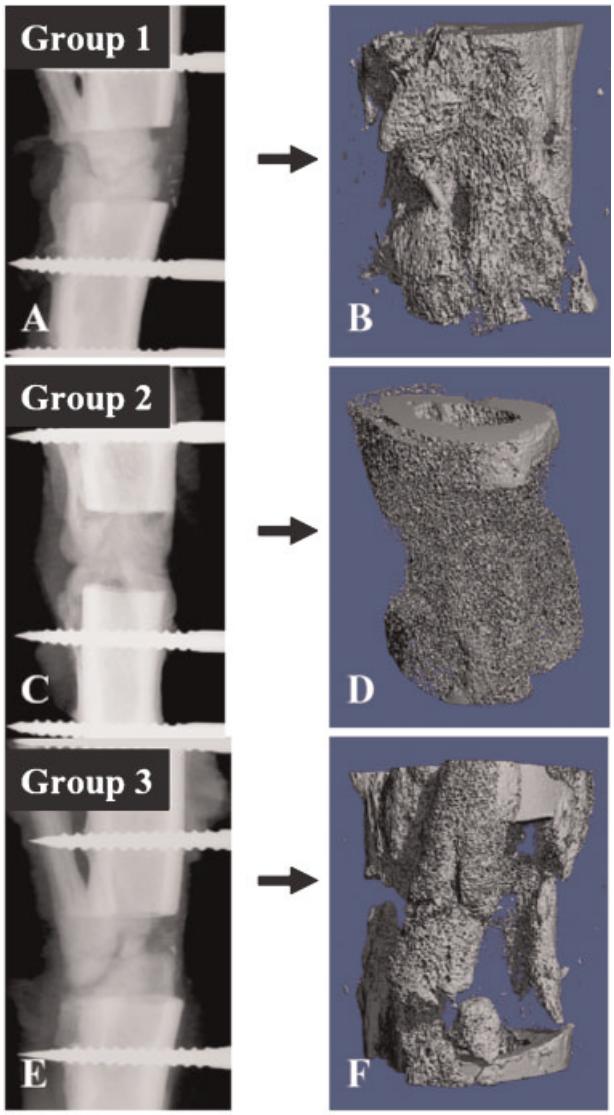

Figure 2. The three-dimensional (3D) image of the entire regenerate was taken using microCT. Left panel show the radiographs (A, C, E) of the representative regenerates of three groups at second week postlengthening, and right panels show the 3D microCT images (B, D, F) of the corresponding same specimens. The quality of newly formed bone was much better in Group 2 (D) as the callus was well organized and evenly distributed along the distraction force, whereas the newly formed bone was less well organized and uneven in Group 1 (B) and focal bone defects were frequently seen in Group 3 (E).

bone formation appeared more evenly distributed in the distraction gaps in the TP508 PPF/PLGA microparticle group. TP508 encapsulated in PPF/PLGA microparticles allows a steady release of TP508 during the course of miscrosphere breakdown, and this provides a continuous supply of TP508 in the local tissues. Pharmacokinetic studies with ^{125}I-labeled TP508[10] indicate that more than 90% of the labeled peptide radioactivity is excreted within 24 h in vivo; thus it is likely that the direct effects of TP508 occur within the first 24 hours of its application when it was delivered in saline. TP508 encapsulated in PPF/PLGA microparticles was protected from its rapid degradation and was able to retain its biological activities for a prolonged period till its release from the PPF/PLGA microparticles.[19,20] In the present study, during the course of 2 weeks after TP508 was injected in PPF/PLGA microparticles there was continuous TP508 release, whereas the bioactivities of TP508 was shortlived in the group receiving TP508 in saline, and this may explain the difference in regenerate histology between the two groups observed. The unique slow releasing characters of PPF/PLGA microparticle encapsulated TP508 may represent a biologically active thrombin peptide release during degradation of the fibrin clot, and functions as an upstream effector that triggers and regulates the expression of other growth factors and enzymes during soft tissue and bone repair.[1,4,8,10,11]

A previous study on rat fracture healing had demonstrated that TP508 stimulated the rate of healing in both young and old rats.[7] In wound healing models, application of TP508 leaded to increase neutrophil accumulation and activation, vascularization, and collagen and matrix production.[1,10] A recent microarray study has demonstrated that genes involved in osteoblast differentiation (such as Cbfa-l), matrix synthesis (such as collagen types I and II), and angiogenesis (e.g., VEGF) were all upregulated in the TP508-treated rats at various time points during fracture healing; TP508 significantly induced expression of early growth factors, inflammatory response modifiers, and angiogenesis-related genes.[11] In

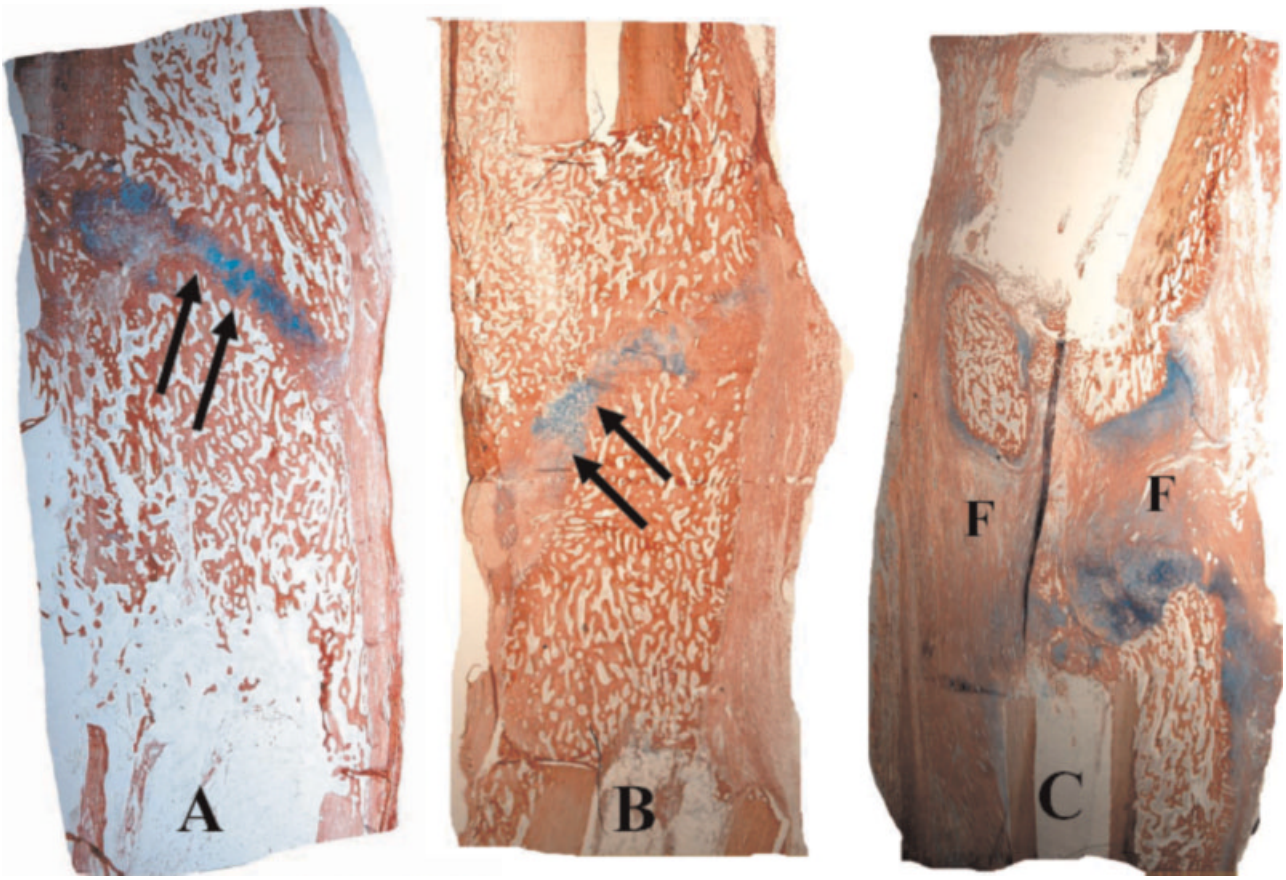

Figure 3. Histology sections representative of the typical appearance of the distraction regenerates from the three experimental groups. (A) Group 1, showing that the regenerate was mainly consisting of well-organized woven bone, with signs of callus remodeling and cartilaginous tissues (arrows). The bone formation was less even throughout the regenerate. (B) Group 2, showing that the regenerate was nearly consolidated completely. The newly formed callus was in parallel with the distraction force and evenly distributed in the distraction gap, and there was a small amount cartilaginous tissues (arrows) present at the center of the regenerate. (C) Group 3, showing that the regenerate contained focal defects with considerable amounts of fibrous (F) and cartilaginous tissues (C), suggesting the bone consolidation was long way to go. (A–C) Sirius red/Alcian blue staining; original magnification, ×15. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

addition, TP508 has also showed the ability of repair of full-thickness articular cartilage defects.[8] A previous study has demonstrated a significant increase in blood vessels in TP508 treated full-thickness skin incisions.[1] TP508 stimulated angiogenic sprouting to an extent similar to or greater than the potent angiogenic factor VEGF. The increased sprouting activity stimulated by TP508 was VEGF dependent but did not involve an increase in VEGF mRNA expression above baseline levels; TP508 acts early in angiogenesis and directly on microvascular cells to accelerate sprouting.[6,27] Human endothelial cells and osteoblastic cells are chemotactic to TP508.[28] When applied locally in slow releasing form, TP508 may enhance angiogenesis and neovascularization of the injured tissue.[1,3,4,6,27]

Osteogenic peptides such as TP508 offer an advantage over proteins—for example, rhBMP-2—as they are often less costly to produce and less likely to lose bioactivity during storage and delivery due to their short, linear structure.[19,20] TP508 encapsulated in PPF/PLGA microparticles allows its efficient delivery in vivo; a carrier that can be implanted or injected directly into a defect site, resulting in localized drug delivery and reduces possible toxic systemic effects. In addition, the rate of release of the entrapped TP508 can be controlled by varying the preparations of PPF/PLGA microparticles to meet the temporal requirements of the wound-healing response.[19,20]

There are limitations in this study and future improvement of the study design and evaluation should include a control group with PPF/PLGA

microparticles incorporated with vehicle solution, mechanical testing of the regenerates, and detailed histomorphometry analysis of the regenerates.

In conclusion, we have demonstrated that a single injection of small amount of TP508 (300 μg) at the end of lengthening phases has significantly enhanced bone consolidation process in a rabbit model of distraction osteogenesis. The delivery of TP508 in PPF/PLGA microparticles appears to lead to a better quality bone formation over the saline delivery, and further examinations are needed to confirm if PPF/PLGA microparticles may be desirable drug delivery form in augmenting bone formation.

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