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Bone formation is enhanced by thrombin-related peptide TP508 during distraction osteogenesis

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Abstract

The thrombin-related peptide, TP508, has been shown to promote soft tissue healing and fracture repair. One possible clinical application of TP508 is to accelerate bone regeneration during distraction osteogenesis, which is a lengthy procedure involving significant complications. In this study, we tested the ability of TP508 to accelerate the consolidation phase of distraction osteogenesis in a rabbit model of leg lengthening. Twenty-three rabbits had left tibiae lengthened for 1 cm over a period of 6 days. TP 508 (0, 30 and 300 μg in 300 μl saline) was injected into the distraction gaps at the beginning and the end of the lengthening phase, and all the animals were killed 2 weeks after lengthening. By the end of experiment, more animals in the TP508 treated groups had complete bony union of the distraction gaps when compared to the saline treated group. pQCT examination of the regenerates demonstrated a significantly greater bone mineral density (BMD) in the TP508 treated groups relative to the saline control group, but no statistical difference in the BMD was found between the two dosages of TP508. Bone consolidation and bone remodeling was far advanced in the TP508 300 μg treated group, and the regenerates mainly consisted of well-vascularized woven bone. In contrast, in the group that received the 30 μg TP508 treatment, focal bone defects and discontinuities of the new cortices were evident in some but not all animals. In the saline control group a majority of the animals showed large amounts of fibrous and cartilaginous tissues in the regenerates, and none of the regenerates had completed consolidation. This study has demonstrated that local application of TP508 enhanced bone formation and consolidation during distraction osteogenesis in the rabbit. The findings indicate that TP508 may be useful in promoting osteogenesis in situations when augmentative treatment for bone formation and consolidation are needed.

Keywords: Thrombin-related peptide; TP508; Distraction osteogenesis; Bone consolidation; Rabbit

Introduction

Induction of new bone formation by means of an osteotomy, followed by fixation with an external fixator and subsequent controlled elongation of the callus, is termed distraction osteogenesis. Distraction osteogenesis has widespread clinical applications in the treatment of bone defects, limb deformities and fracture nonunions [4,5,11,12]. In clinical practice, a long duration of the bone consolidation phase is usually encountered and means for augmentation of bone consolidation during distraction osteogenesis is often needed [11,12].

Bone formation in distraction osteogenesis resembles a continuous fracture and healing situation. Normal fracture healing usually starts with a hematoma, which contains mainly blood cells at early stages. The organization of the hemotoma involves blood clotting, activation of platelets, release of many cytokines, growth factors, hormones and synthesis of new extracellular matrix components. A number of endogenous growth factors and cytokines including TGF-βs and BMPs have been identified as possible modulators of wound healing and fracture repair [16]. One mitogenic factor, thrombin, which is present in the very early stage at the sites of tissue injury, may have been grossly overlooked. Thrombin's important role in formation of fibrin clots and platelet activation is well documented. It is also known to interact with many cell types involved in both early and late stages of tissue repair/healing [19]. When clots dissolve, thrombin fragments activate specific receptors found on many cells to initiate healing [18,19].

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There are two types of thrombin receptors that act independently: proteolytically activated and non-proteolytically activated receptors [18]. TP508, also known as Chrysalin® (Chrysalis BioTechnology, Inc., Galveston, TX, USA), a synthetic 23 amino acid peptide, represents a receptor-binding domain of the human enzyme prothrombin. TP508 mimics thrombin effects in accelerating initiation of wound healing by interacting with non-proteolytically activated receptors without affecting the blood clotting activity of thrombin [18,19]. In a closed diaphyseal fracture model, a single injection of 1 μg TP508 accelerated the rate of bone healing in young and old animals relative to controls [14]. When TP508 was formulated in biodegradable controlled-release microspheres in rabbit ulnar segmental defects, these critical-size segmental defects showed advanced stages of complete regeneration [15]. Therefore, one possible application of TP508 is to enhance bone formation and regeneration during processes such as distraction osteogenesis. Thus, the aim of this study was to test the ability of TP508 to accelerate the consolidation phase of distraction osteogenesis in a rabbit model of leg lengthening.

Materials and methods

Animal model of distraction osteogenesis and experimental groups

All animal experimental procedures were approved and performed under the control of the guidelines for Animals (Scientific Procedures) Act 1986, British Home Office. Mid-tibial osteotomies were performed in 30 adult male NZW rabbits (age 24 weeks, body weight 2.6–3.5 kg; mean 2.68 ± 0.26 kg), with the tibiae stabilized with external fixators as previously described [6,7]. After a 7-day latency period, once daily lengthening was initiated at rate of a 1.4 mm/day for 6 days.

Among the 30 experimental animals, seven rabbits died or were euthanised before the experiment was completed, because of anaesthetic death ($n = 2$), pinhole fracture ($n = 3$) and soft tissue complications ($n = 2$). These animals were excluded from the experimental groups. The remaining 23 rabbits were randomly divided into three experimental groups, each group consisting of at least seven rabbits. In all groups, TP508 or saline were administered immediately via percutaneous injection into the distraction gap under X-ray guidance at the beginning and the end of the lengthening phase. Group 1 ($n = 8$) received injections of 300 μl saline containing 300 μg TP508; Group 2 ($n = 8$) received injections of 300 μl saline containing 30 μg TP508; Group 3 ($n = 7$) received injections of 300 μl saline alone. The first injection (100 μl) was given at the center of the distraction gap; the second and third injections of 100 μl were injected 0.3 cm distal and proximal to the central (first) injection point. During the experiment period, the animals were free to weight-bear on the operated leg. All animals were sacrificed at 2 weeks post-lengthening. 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.

Radiographic examination

Serial radiographs were taken at the day of surgery, end of lengthening, and 1 and 2 weeks post-lengthening, using a high-resolution digital radiography system (Faxitron MX-20 with DC-2 option, Faxitron X-ray Corporation, Illinois, USA). The exposure condition was 32 kV, 10 ms at 1× magnification. The percentage areas of the distraction gap occupied by new bone was scored by two independent and blinded observers according to the percentages of the distraction gaps filled by new bone. The percentage area of the distraction gap occupied by new bone was graded from 1 to 4 (Table 1) on the radiographs at 2 weeks post-lengthening. 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.

Table 1
Mean scores for the final X-rays

Group	Mean X-ray scores	Number of fully united animals
1. TP 508 300 μg	3.25*	4/8 (50%)
2. TP 508 30 μg	2.71	3/8 (37.5%)
3. Saline	2.50	2/7 (28.6%)

* $p < 0.05$, with Groups 2 and 3.
1. <25% distraction gap filled with new bone.
2. 25–50% distraction gap filled with new bone.
3. 50–75% distraction gap filled with new bone.
4. >75% distraction gap filled with new bone.

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, USA) 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 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.

Histological examination

After the pQCT examination, the samples were fixed in 10% buffered formalin for 48 h 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. 7 μm sections were cut at and stained with routine hematoxylin and eosin (HE) and Alcian blue/Sirius red. Alcian blue/Sirius red staining, following de-paraffin, 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 9, Chicago, Illinois, USA). Data from each group were tested by Mann–Whitney U tests and differences were considered significant at $p < 0.05$.

Results

General observations

All animals lost an average of 0.23 kg body weight during the course of the experiment. Average weight loss

was 0.23 kg in Group 1; 0.27 kg in Group 2 and 0.22 kg in Group 3. During the first week post-lengthening, animals in Group 1 appeared to be more calm, all their skin wounds healed, and none developed skin or pinhole infection and no soft-tissue swelling on the operated legs was seen. In contrast, by the end of 1 week post-lengthening, the skin wound did not heal completely in 2 out of 8 rabbits in Group 2 and 3 out 7 rabbits in Group 3; signs of swelling and tenderness at the operated legs were found in half of the animals in both Groups 2 and 3 and these animals did not bear weight on the operated legs. During the second week post-lengthening, 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 9.0 ± 0.6 mm. There was no difference found on the radiographs at the end of lengthening (Fig. 1). By the end of the first week post-lengthening, radiographs showed that there was more bone formation in the TP508-treated groups than in the saline control group (Fig. 1). At second week post-lengthening, Group 1 had significantly greater mean X-ray scores compared to Groups 2 and 3 (Table 1, $p < 0.05$, Mann–Whitney U test). At 2 weeks post-lengthening, the radiographic signs of cortical continuity were observed in 4/8 rabbits

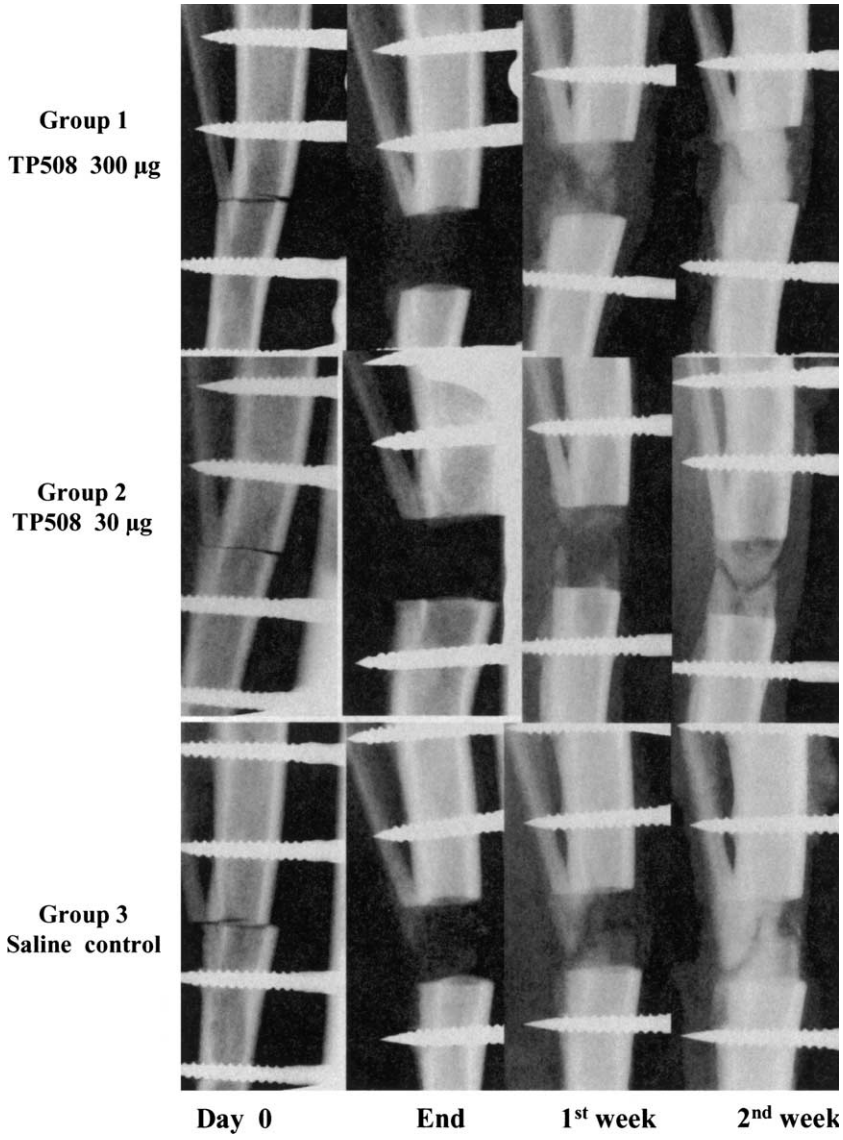

Fig. 1. Representative radiographs of all experimental groups at day 0, end of lengthening, 1 and 2 week post-lengthening. There is a significant increase in callus formation in the TP508 treated groups compared with the saline group at 1 and 2 week post-lengthening. Most advanced bone formation and consolidation is seen in the group with TP508 300 μg treatment. At second week post-lengthening, although bony unions were seen in all groups, radiolucent regions representing focal defects in the distraction regenerates were frequently seen in Groups 2 and 3, and formation of a new cortex was evident only in some animals in Group 1.

in Group 1, in 3/8 rabbits in Group 2, and in 2/7 of the Group 3 rabbits (Fig. 1 and Table 1). Radiolucent regions representing focal defects in the distraction regenerates were frequently seen in Groups 2 and 3 (Fig. 1).

pQCT results

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

Histology results

At 2 weeks post-lengthening, the distraction regenerates of half of the animals in Group 1 (TP508 100 μg) had completed consolidation. The distraction gaps consisted mainly of woven bone, with signs of neocorticalization and callus remodeling (Fig. 2A). In Group 1, the newly formed bones were highly cellular (Fig. 3A) and vascular, with numerous small vessels and capillaries observed (Fig. 3B). The remaining half of the animals in Group 1 had various degrees of consolidation, some had cartilaginous and fibrous tissues in the regenerates, but the amounts were less than those of the animals with non-consolidated regenerates in Groups 2 and 3 (not shown). In Group 2, less than half of the animals' regenerates had shown complete consolidation. Of those animals whose regenerates did not consolidate, there were often fibrous and cartilaginous tissues observed at the centers of the regenerates (Fig. 2B). The newly formed callus in the regenerates of Group 2 were less proliferative and vascular than those seen in Group 1 (Fig. 3C), however, the fibrous tissues at the centers of the regenerates were usually proliferative and mainly intramembranous ossification took place in these regions (Fig. 3D). In Group 3 (saline control), less than a quarter of the animals had the regenerates consolidated fully. In Group 3, even in the regenerates considered consolidated on X-ray; focal defects with considerable amounts of cartilaginous tissues and signs of endochondral ossification were frequently seen (Figs. 2C and 3E). There were mixtures of fibrous, cartilaginous and bony tissues in most of the regenerates in Group 3 (Fig. 3E). It has been noticed that some fibrous tissues in the regenerates of Group 3 containing typical spindle cells with dense connective tissue appearances were not as cellular as the ones seen in Group 2 (Fig. 3F).

In comparison, bone consolidation and remodeling was most advanced in the Group 1, where half of the

Table 2
Mean BMD of the regenerates (mg/cm^2)

Group	BMD (mean ± SD) (mg/cm^2)	p value (Mann–Whitney U test)
1. TP 508 300 μg	495.48 ± 60.94	0.045 (with Group 3)*
2. TP 508 30 μg	477.44 ± 39.19	0.036 (with Group 3)*
3. Saline	426.78 ± 36.77	0.55 (Groups 1 and 2)

* $p < 0.05$, Mann–Whitney U test.

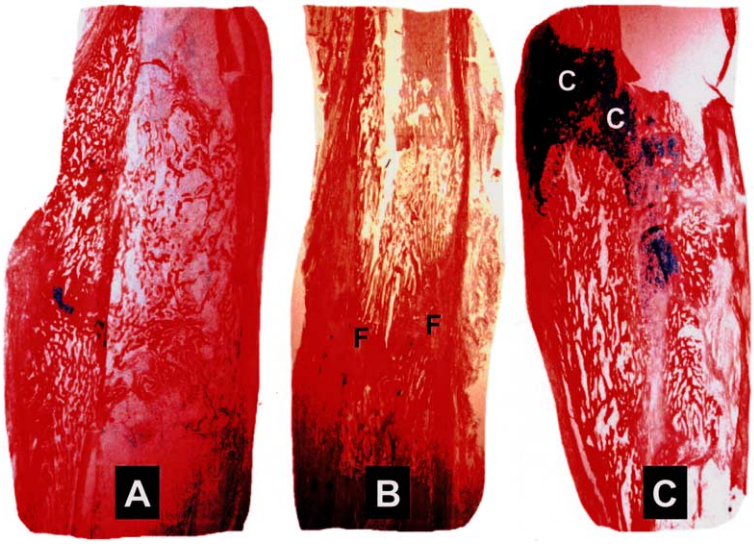

Fig. 2. Histology sections representative of the typical appearance of the distraction regenerates from the three experimental groups. (A) Group 1, showing that the regenerate has completed its consolidation, mainly consisting of well-organized woven bone, with signs of neocortilization and callus remodeling. (B) Group 2, showing that the regenerates did not fully consolidate. There are fibrous (F) and cartilaginous tissues present at the center of the regenerates. (C) Group 3, showing that the regenerate contains frequent focal defects with considerable amounts of cartilaginous tissues (C) and signs of endochondral ossification, suggesting the bone consolidation is not yet completed. A–C, Sirius red/Alcian blue staining, original magnification ×15.

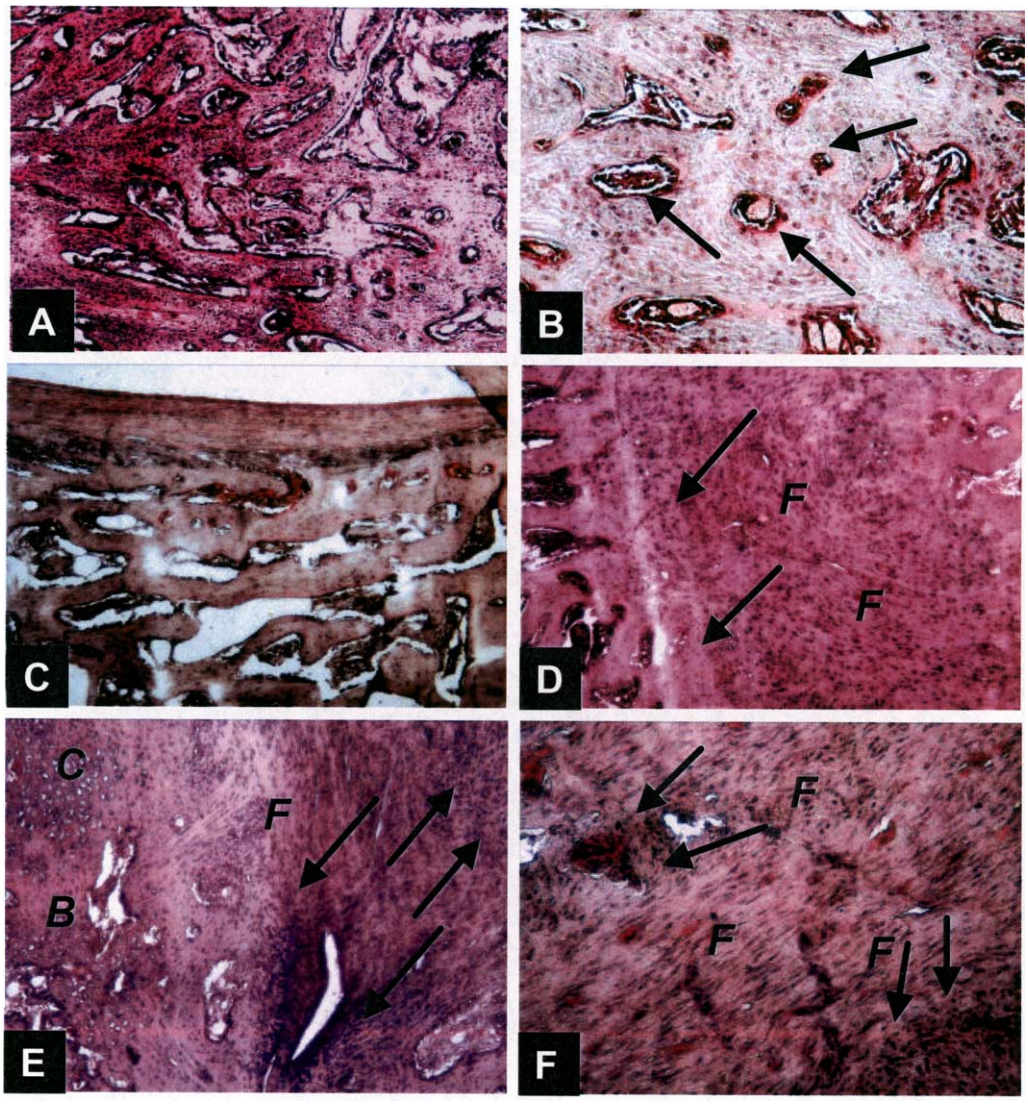

Fig. 3. Histology images representative of the three treatment groups. (A) The greater amount of bone formation was found in Group 1, the newly formed bones were highly cellular. (B) The regenerate from Group 1 was highly vascular, with numerous small vessels and capillaries incorporated in the newly formed bone. (C) The newly formed callus in the regenerate of Group 2 was less proliferative and vascular than those seen in Group 1. (D) The fibrous tissues (*F*) at the centers of the regenerate of Group 2 were usually proliferative. Mineralization was mainly through intramembranous ossification pathway (arrows). (E) There were mixtures of fibrous (*F*), cartilaginous (*C*) and bony (*B*) tissues in the regenerates in Group 3. Inflammatory cells were also present in the regenerate (arrows). (F) Fibrous tissues (*F*) in the regenerate of Group 3 contained typical spindle cells with dense connective tissue appearances, were not as cellular as the ones seen in Group 2, note the presence of inflammatory infiltrations (arrows). A–F, H&E staining; A, C–E, original magnification ×100; B and F, original magnification ×400.

animals' distraction gaps were completely united with woven bone, new cortex and bone marrow cavity formation evident in some animals (Fig. 2A). The number and maturity of blood vessels in the distraction regenerates were also found to have increased in sections of the TP508 treated animals, more so in the high dose group (Fig. 3B). Focal bone defects and partial discontinuities of the new cortices were still evident in some animals in Group 2 (Figs. 2B and 3D) and in majority of animals in Group 3 (Figs. 2C and 3E, F). Large amounts of fibrous and cartilaginous tissues were only visible in Group 3 (Figs. 2C and 3F) at 2 weeks post-lengthening.

The number of inflammatory cells was greatly reduced in the TP508 treated groups. Samples from Group 1 showed almost no sign of inflammation. Inflammatory cells (mainly lymphocytes) were still found in Groups 2 and 3 (Fig. 3E, F), but the number of these cells was less in Group 2 relative to Group 3.

Discussion

Distraction osteogenesis is a unique process in which bone formation occurs in parallel with rapid bone remodeling [1,3–5,11]. This technique is widely used

clinically for the treatment of many challenging orthopedic conditions, such as correction of congenital deformities and limb reconstruction following tumor resection [4,5,11]. Despite the promising results, a prolonged period of external fixation is usually required to allow sufficient bone consolidation before the fixator is removed. Complications, such as refracture, cause tremendous pain and morbidity [11,12]. In this study we have demonstrated that significant increases in bone mineral density of the distraction regenerates were achieved by administration of TP508 in a rabbit model of distraction osteogenesis.

The rabbit model of leg lengthening is a well-established model of distraction osteogenesis and has been used extensively to study various aspects of bone regeneration during distraction osteogenesis [3,6–9]. Previously reports using this model have shown that the optimal rate of lengthening is between 0.7 and 1.3 mm, twice daily lengthening [6,7]. However, when lengthened at a higher rate (>1.3 mm/day), the rate of consolidation is decreased, the quality of bone formation is poor and there is a high incidence of soft tissue complications [7,9,17]. In this study, we deliberately chose a fast rate of lengthening (1.4 mm/once a day) to mimic clinical situations of poor bone formation during distraction osteogenesis. The total lengthening ($\sim$1 cm) was less than 10% of the original tibial length, which was well tolerated by all the animals.

In the present study we have shown that the consolidation of the distraction regenerates was enhanced when TP508 was injected into the distraction gap at the early stages of distraction osteogenesis. The increase in BMD correlates with histological findings of more advanced stages of bone formation. The decreased inflammation in the animals receiving TP508 treatment suggests that the inflammatory stage of healing has already passed and the regenerates have progressed into a more proliferative or maturation phases. The dose of TP508 used in the present study was based on our previous fracture studies data [14,21], and the dose tested was approximately 10 and 100 μg/kg per rabbit. Although the two doses tested all showed positive effects on bone consolidation, the animals that received the higher dose of TP508 had better healing when compared with the low-dose group from the radiographic and histological examinations. A further dose ranging study or alternative formulations may be needed for subsequent animal trials or clinical studies.

We have chosen two time-points for injection based on our understanding of the biology of distraction osteogenesis. At day 1 of lengthening, the sudden widening of the fracture gap induced an acute tissue injury to the organized connective tissues in the gap; the injection of TP508 at this point may have helped to recruit inflammatory cells and other reparative cells into the distraction gap. When the daily lengthening continued, fresh trauma was repeatedly induced in the gap tissues where the active repair process was also ongoing. By the end of lengthening, a further injection of TP508 was given to boost the repair process in order to shorten the consolidation phases. One advantage of using percutaneous injection to deliver TP508 is that it allows a precise dose of the peptide to be delivered the desired areas and makes repeated deliveries possible. Pharmacokinetic studies with ^{125}I-labeled TP508 [20] 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 h of its application.

The early phase of normal fracture healing is characterized by the influx of inflammatory cells (neutrophils, monocytes and T-lymphocytes) from the circulation to the sites of fracture. These inflammatory cells are then activated at the injured sites, and play multiple roles in tissue healing, including releasing proteases for wound debridement, phagocytosing debris, and secreting various cytokine and growth factors which, in turn, orchestrate the activity and interactions of other cell types during bone formation and remodeling. Therefore, we have chosen to apply TP508 at the early phases of distraction osteogenesis in order to induce maximal effects on the subsequent cascades of other cellular events.

TP508 may represent a biologically active thrombin peptide release during degradation of the fibrin clot, and function as an upstream effector that triggers and regulates the expression of other growth factors and enzymes during soft tissue and bone repair [18,20,21]. Therefore, signals induced by application of TP508 into the wounded areas, despite its short half-life could be amplified to initiate or accelerate the timing of the body's normal healing responses, and accelerate the subsequent cellular events that lead to an enhancement in bone formation and consolidation during distraction osteogenesis. In concurrence with the findings in the present study, a previous study on rat fracture healing had demonstrated that TP508 stimulated the rate of healing in both young and old rats [14]. In soft tissue wound healing models, TP508 has been shown to increase (1) neutrophil accumulation and activation, (2) vascularization, and (3) collagen and matrix accumulation [2,20]. Whether these processes are also responsible for the increased rate of healing observed in the present study is not yet known, however, the temporal and dynamic processes of healing in both hard and soft tissue share many similarities.

To understand the cellular and tissue level mechanisms underlying the acceleration of fracture healing and bone regeneration by thrombin peptides, we have shown in a recent experiment that genes involved in osteoblast differentiation (Cbfa-l), matrix synthesis (collagen type II), and angiogenesis (VEGF) were all upregulated in the TP508 treated rats at various time points during fracture

healing [21]. In addition, TP508 formulated in controlled release microspheres has also showed repair of full-thickness articular cartilage defects [10].

Part of the TP508 mechanism may be related to its enhancement of neovascularization of the injured tissue [2,13,20]. In the present study, we have found an increase in blood vessel number and maturation in the distraction regenerates with TP508 treatments. One previous study has demonstrated a significant increase in blood vessels in TP508 treated full-thickness skin incisions [2]. TP508 has been shown to be angiogenic when assayed on chicken chorioallantoic membranes, and the peptide is chemotactic to human endothelial cells [13]. Revascularization of an injured tissue is a dynamic process that begins with microvascular endothelial cells to migrate into the provisional matrix and lead to vascular buds formation in conjunction with the process of bone formation, maturation and remodeling. Thus, the effects of exogenously added TP508 may therefore help acceleration of initial events in recruiting inflammatory, endothelial and osteoblastic cells into the distraction gaps at the early phases of bone formation. Application of TP508 to the newly formed bone matrix may also enhance angiogenesis and promote revascularization and bone remodeling during the consolidation phase of distraction osteogenesis.

In conclusion, TP508, a thrombin-related peptide, may represent a new class of bioactive molecules that regulate early stages of repair/healing, by initiating the body's entire natural growth factor and cytokine cascade. Since TP508 is synthetically manufactured, it can be produced efficiently at low cost relative to recombinant growth factors. Based on clinical evaluation, TP508 may be an effective product for augmenting bone formation in conditions that may otherwise lead to the prolonged treatment or poor clinical outcome, such as limb lengthening, spine fusion and high-energy fracture management.

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