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Thrombin peptide (TP508) promotes fracture repair by up-regulating inflammatory mediators, early growth factors, and increasing angiogenesis

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Abstract

Previous studies have shown that a single injection of thrombin peptide (TP508) accelerates fracture repair in a closed rat femoral fracture model. The present study was conducted to elucidate the molecular mechanisms of TP508 action using Affymetrix genome-scale profiling and to link early gene expression changes to fracture histology and bone strength changes. Treatment of femoral fractures with TP508 accelerated fracture repair as determined by destructive torsion testing. Blinded histological analysis demonstrated that TP508-treated fracture callus had a significant increase in blood vessels relative to the controls. Gene array analysis showed that TP508 significantly induced expression of early growth factors, inflammatory response modifiers, and angiogenesis-related genes. This study therefore suggests that TP508 promotes fracture repair through a mechanism that involves an increased induction of a number of growth factors, enhanced expression of inflammatory mediators, and angiogenesis-related genes.

Keywords: Fracture repair; TP508; Gene expression; Early growth factors; Angiogenesis; Microarray analysis

Introduction

Fracture repair is a complex physiological process, which can be enhanced through therapeutic approaches. The fracture repair process is divided into five distinguishable stages as described by Einhorn: the initial hematoma and inflammation, intramembranous bone formation, angiogenesis and chondrogenesis, endochondral bone formation, and bone remodeling [12,24,38]. At the initial phase, inflammatory cells, macrophages, and degranulating platelets infiltrate into the fracture site from peripheral blood. Various growth factors and cytokines released from these cells play key roles as initiators of the fracture repair process resulting in osteoprogenitor cell differentiation and proliferation, and angiogenesis [1,4,11,13,18]. A therapeutic molecule may alter any of these stages, potentially enhancing the overall fracture repair process.

Angiogenesis is an essential part of fracture repair that may be initiated as early as the hematoma and inflammatory stage [17,42]. Inadequate or inappropriate vascularity is associated with decreased bone formation, and development of delayed and mal-unions [7,34]. In addition, administration of an angiogenesis inhibitor prevents femoral fracture repair in rats [19]. Angiogenesis is regulated by many growth factors induced in

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response to injury, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) [4,21,32]. Therefore, treatment with angiogenic molecules may be expected to promote early angiogenesis and accelerate later stages of fracture repair.

A number of recombinant growth factors, regulatory peptides, and small molecules have been tested in recent years for potential efficacy in stimulating fracture repair [2,20,25,29,36,39,41]. Among the above molecules, recombinant human bone morphogenetic proteins (BMP), including BMP-2, BMP-4, and BMP-7, have shown significant enhancement of the repair process in different animal models of fracture repair [10,35,44]. Although recombinant growth factors have shown effectiveness, the use of peptides or small molecules is a promising alternative due to ease of synthesis, stability, and lower cost.

The thrombin related peptide, TP508 (Chrysalin®), is a synthetic 23 amino acid peptide representing the natural sequence of the receptor-binding domain of human thrombin (prothrombin amino acids 508–530). This peptide was initially identified by its ability to compete with thrombin for binding to a high-affinity thrombin receptor on fibroblasts and to generate receptor occupancy-dependent mitogenic signals [16]. Subsequently, a number of preclinical studies demonstrated a potential therapeutic role of TP508 in tissue repair. A single topical application of TP508 enhanced repair of full dermal wounds [9], and accelerated both closure of full-thickness excisional wounds in normal rat skin [40] and dermal excisions in rat skin with surgically induced ischemia [28]. Interestingly, in each of these dermal wound studies, TP508 acceleration of the repair process was associated with early recruitment of inflammatory cells to the site of injury and enhanced early angiogenesis [27].

More recently, the application of TP508 has been evaluated in orthopedic tissue repair models. A single fracture site injection in a rat closed femoral fracture model [36,39] showed an increase in bone strength of approximately 40% in 2-month-old rats, and 25% in 8-month-old rats. TP508, delivered in controlled release poly-lactoglycolide (PLGA) microspheres, stimulated bone formation in rabbit segmental defects [37]. Interestingly, TP508 delivery to rabbit cartilage defects using the same microspheres enhanced repair of full thickness articular cartilage defects in rabbits [22]. These studies show that TP508 enhances musculoskeletal tissue repair, although its underlying mechanisms are unknown.

In this study, we investigated the molecular mechanisms of TP508 action in enhancing fracture repair. Since TP508 represents a receptor-binding domain of human thrombin, and thrombin plays a critical role in hematoma and angiogenesis during soft-tissue repair, we hypothesized that TP508 accelerates fracture repair by modulating the expression of early response and angiogenesis-related genes, such as inflammatory cytokines and growth factors. We tested the hypothesis by performing histology and mechanical testing at three and four weeks post-fracture, respectively, and comprehensive gene expression studies at the early stage of fracture repair.

Materials and methods

Animal model of fracture

All animal experimental procedures were approved and performed under the guidelines of the Institutional Animal Care and Use Committee (IACUC). Ten-month-old male retired breeder Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 450–550 g were randomly divided into three groups in this study. Each rat was subjected to a closed transverse fracture on its right femur as described by Bonnarens and Einhorn [5]. Briefly, animals were anesthetized with intramuscular injection of ketamine and xylazine at 120 mg and 20 mg respectively per kg body weight. A small skin incision was made in the right knee, and the patella was deflected. A 1.1 mm diameter hole was drilled into the intercondylar notch, and a Kirshner wire (1.1 mm diameter) was inserted into the medullary canal. After closing the knee joint, a mid-diaphyseal fracture was induced using a three-point bending device driven by a dropped weight. Fractures were verified via contact radiograph using the Hewlett Packard Model #43855-A Faxitron Closed X-ray System. One hour after fracture, TP508 at doses of 0 (saline control), 1, and 10 μg in 100 μl saline was percutaneously injected into the fracture site of each rat in each of the respective groups. The rats were allowed to bear weight as tolerated after recovering from anesthesia. At predetermined time points post surgery, each rat was anaesthetized with isoflurane and killed with 2 ml Euthasol (containing Sodium pentobarbital, Delmarva Labs, Midlothian, VA) via intraperitoneal injection. The right femurs were harvested as required for the different procedures described below.

Biomechanical testing

This experiment included three groups: control (saline), 1 μg and 10 μg TP508 treated groups. Twelve animals were used for each group at each time point. A total of 108 animals were sacrificed at 2, 3 and 4 weeks post-fracture. Femurs were carefully dissected free of soft tissues, wrapped in 0.9% saline soaked gauze, frozen and retained at −20 °C for destructive torsional testing. Before testing, the exposed ends of the fractured femurs were embedded in Woods metal (Cerrobend alloy, melting temperature 70 °C). Testing was performed with a custom DC servomoter controlled torsion testing apparatus with a deformation rate of approximately 550° or 10 rad/s as described [20]. Biomechanical results are reported as maximum torsional strength [8,30]. Statistical analysis was performed by ANOVA on Ranks (Kruskal–Wallis).

Histology analysis for blood vessel formation

This experiment also included three groups: control, 1 μg and 10 μg TP508 treated groups. Four animals were used in each group and all animals were sacrificed at 3 weeks post-fracture. Fractured femurs were radiographed using the Faxitron, then fixed in 10% buffered formalin, and subsequently demineralized in 5% nitric acid. Pins were removed from the bones before embedding and sectioning. Fixed and decalcified tissues were dehydrated in a series (70%, 90%, 95%, and 100% ×2) of ethanol concentrations, transferred to xylene and embedded in paraffin. Five-micron thin paraffin sections were cut longitudinally and stained using Masson's Trichrome.

Blinded histological analysis of blood vessel formation was performed as follows. Digital images of 12 animal fractures, 4 per group and randomly coded, were captured using a Leica DMLB microscope