Local Application of a Proteasome Inhibitor Enhances Fracture Healing in Rats

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Introduction: The fracture healing process involves multiple stages of repair and the coordinated participation of multiple cell types, which is regulated by systemic and local factors. Bone morphogenetic proteins (BMPs) are important physiologic mediators of fracture repair (1). Proteasome inhibitors (PSIs) have been reported to induce osteoblastic differentiation and enhance bone formation through the BMP2 pathway (2, 3). We therefore hypothesized that these inhibitors might show a beneficial effect on acceleration of fracture healing.

Methods: In vitro osteogenic differentiation: Bone marrow derived (BM) and periosteum-derived (PO) progenitor cells were obtained from 8-week-old Sprague-Dawley (S-D) rats femurs and treated them with PSI (proteasome inhibitor 1: PS1) in vitro monolayer culture. The cells were treated with PS1 (0, 5, or 25 nM) in osteogenic medium containing 2.5% FBS, 100 nM dexamethasone, 5 mM β-glycerophosphate and 100 μg/ml ascorbic acid phosphate for 4 days. Alkaline phosphatase (ALP) activity was measured.

In vivo fracture study: A closed femoral fracture was created, following intramedullary pinning in 8 week old S-D rats (Fig. 1A). Rats were treated with a local injection with vehicle or PS1 diluted in 75% DMSO/PBS. Groups (n=9) included 1) vehicle; 2) PS1 low-dose (PI-L: 2.5mg/kg); 3) PS1 high-dose (PI-H: 10mg/kg) receiving treatment at day 0 and 7 after fracture. Radiographs were taken at day 0, 2 and 4 weeks after procedure, and fusion status was scored (0-7 pts.). Callus area was also measured using lateral radiographs (Fig. 1B). Animals were euthanized at 4 weeks, and evaluated with μCT and biomechanical testing. Fracture callus was evaluated by μCT analysis, excluding original cortices (Fig. 2A). Mechanical strength was assessed by three-point bending. To evaluate the fracture healing process at early time-points, the vehicle group and the PI-H treatment group were evaluated by histology (Safranin-O and Fast green) at one and two weeks post-fracture (n=4). Student’s t-test or one-way ANOVA was used for statistical analysis.

Results: In vitro osteogenic differentiation: The ALP activity in both BM and PO cells at day 4 was significantly enhanced by PS1 treatment.

In vivo fracture study: In radiological analysis at week 2, compared with the vehicle-treated group, there was more apparent initial bridging of the fracture gaps with PSI treatment, particularly in the PI-H group (Fig. 1C). At week 4 post-fracture, complete callus bridging could be observed in the treatment groups, and the original cortices also started bridging (Fig. 1D). However, bridging of the callus gap was not yet substantial in the vehicle group. The radiographic score showed a dose-dependent increase at both 2 weeks and 4 weeks post-fracture (Fig. 1E). Quantitative analysis of callus formation also showed a significant increase in the callus area in the PSI treatment groups at 2 weeks, although the differences diminished at 4 weeks with the start of the remodelling process (Fig. 1F).
In μCT analysis, no significant differences in callus volume were observed at 4 weeks post-fracture. However, there was an increase in BMD in the groups treated with the PSI when compared with the vehicle group, with statistically significant difference in the PI-H group (Fig. 2 B, C).

In biomechanical testing, PSI treatment enhanced callus strength (Fig 2 D, E). That is, the force required to break the callus was greater in the PI-H group than in the vehicle group, and in addition, the structural stiffness of the bone was greater in both the PI-L and PI-H groups (Table 2).

In histological sections, PSI treatment accelerated endochondral ossification at the periosteal site. At week 1, stromal fibrous tissue and cartilaginous tissue were observed with a small amount of newly formed woven bone in the callus beneath the periosteum adjacent to the fracture site (Fig. 3A). Quantitatively, the PI-H group showed a trend towards larger cartilaginous and bone (Fig. 3C, D). However, no significant differences were found. At week 2, the fibrous tissue area decreased, and more periosteal woven bone was observed around the fracture site. Active endochondral ossification was present between the cartilaginous tissue and the woven bone (Fig. 3B). Quantitatively, a significantly smaller cartilaginous area was observed in the PI-H group, which instead presented a larger amount of ossified tissue compared with the vehicle treatment group (Fig. 3 C, D), suggesting that endochondral ossification was enhanced by PSI treatment.

**Discussion:** Mesenchymal progenitor cells derived from various sources including bone marrow, periosteum and surrounding soft tissues contribute to fracture healing. In the current study, we isolated mesenchymal progenitor cells from rat bone marrow and the deep layer of periosteum and treated with PSI in vitro. The in vitro osteogenic differentiation was stimulated by PSI treatment in both bone marrow-derived and periosteum-derived progenitor cells. Since those progenitor cells play a critical role during fracture healing process, this result supports the possibility of a beneficial effect of PSI on fracture repair.

When rats were treated with a locally administered PSI, radiographic fusion scores increased at weeks 2 and 4 following femur fracture. The callus area was also significantly increased at week 2 when compared with the vehicle-treated control. The differences in the radiographic callus area did however diminish by week 4, suggesting a treatment-related acceleration in mineralisation and remodelling of the callus. In the μCT analysis, there were no differences between the control and treatment groups in callus volume at week 4. However, the callus density was significantly enhanced in the treatment groups. The stimulatory effect of PSI on fracture repair was further confirmed by biomechanical testing, in which the force required to break the bone and the structural stiffness were enhanced in the PSI treatment groups.

Histology was used to evaluate the process of fracture healing at early time-points. The area of the cartilaginous callus was larger in the treatment group at one week post-fracture, indicating the positive stimulation of callus formation beginning early after fracture healing. At week 2, a decreased cartilaginous area and an increased amount of bone tissue were observed in the fracture calluses in the treatment groups, suggesting that the process of endochondral ossification was accelerated by PSI treatment. These results also confirm the stimulatory effect of local PSI treatment on fracture healing, along with the findings of the radiographic and μCT analyses as well as biomechanical testing.

**References:**


Significance: This is the first report that shows local application of proteasome inhibitor promotes fracture healing.