Enhancement of Fracture Repair by Local Application of VEGF, PTH 1-34 and IL-6

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INTRODUCTION:
Satisfactory fracture healing following trauma, resection after neoplasia or bone disease often entails complex surgical procedures. The aim of the orthopaedic surgeon is to simplify treatments, alleviate pain and enable quick return to daily activities of living. Future technologies for enhancing fracture healing aim at accelerating repair and preventing delayed or non-union fractures. A good vascular network is obligatory for all the different stages of fracture healing, including recruitment of skeletal progenitors, periosteal intramembranous bone formation, remodeling of the cartilage into bone trabeculae in the endochondral ossification stage and remodeling of the primary cancellous bone into lamellar bone. The angiogenic events that accompany the repair process function as a “limiting factor” and are the primary regulatory mechanisms that direct repair. Endothelial cells that are recruited to the injury site by angiogenic growth factors, mostly by VEGF, are responsible for the build-up of a functional blood supply network. We have previously developed a fracture model in rat tibia and have shown with this model that sequential local injections of PTH1-34 followed by IL-6+IL-6sR stimulated chondrogenesis and osteogenesis and improved biomechanical resistance (Rosen et al. Bone vol 41, 2007). The aim of the present study was to test whether VEGF alone or when combined with PTH1-34 and IL-6+IL-6sR enhance the process of fracture healing.

METHODS:
Fracture model in a rat tibia: In anaesthetized rats (Wistar, 250 gr), a hollow wire pin was introduced through the medial aspect of the tibia till the middle of the medullary canal. Closed mid-shaft tibiae fractures were induced by the “Rozen device” that was developed in our laboratory producing anatomically uniform transverse fractures. Following fractures rats were pushed to reach the ankle joint. Rats were free to move with full weight bearing throughout the experimental period. All animal experiments were approved by the Technion Review Board conforming to the laws and regulations of Israel.

Study design: Rats were randomly divided into 4 experimental groups; A) saline injected control; B) PTH1-34+IL-6+IL-6sR; C) VEGF; D) PTH1-34+IL-6+IL-6sR+VEGF. A total volume of 0.2 ml was directly injected under the skin above the fracture site at 11.00 AM. One μg rat PTH 1-34 was injected on days 4, 5, and 6 post-fracture. 40 ng IL-6+100 ng IL-6sR were injected on days 7, 9 and 11 post-fracture and 1.0 μg VEGF was injected daily on days 1-10 post-fracture. Eight rats from each experimental group were sacrificed on days 15 and 30 post-fracture and their fractured bones were analyzed by histology (5-6 micron paraffin sections stained with H&E) and micro-computed tomography (μCT). Five rats from each experimental group were sacrificed at 45 days post-fracture and their fractured bones were analyzed for mechanical resistance to torsion.

μCT: The specimens were scanned at a resolution of 16μm/voxel using a Scanco μCT40 micro-computed tomography system. A threshold corresponding to 25% of the maximum gray value (0.25±2) was applied to distinguish well mineralized tissue from un-mineralized and/or poorly mineralized tissue. The following outcome measures were quantified for each specimen: mineralized callus volume fraction (BV/TV [unitless]), total callus volume (BV), mineralized callus volume (BV normalized by callus length [mm^3]); tissue mineral density (TMD [mg HA/cm^3]). BV was calculated by applying a threshold to exclude un-mineralized and poorly mineralized tissue.

Statistics: A standard 2-factor analysis of variance (ANOVA) was carried out for each outcome measure in order to test for the effects of treatment, time and the interaction between treatment and time (treatment*time). The value of BV was log-transformed prior running the ANOVA, in order to have the distribution of the data satisfy the assumptions on which the ANOVA is predicated. If the effect of treatment or treatment*time was significant (p<0.05), a Tukey post hoc analysis was performed to identify pair wise differences.

Bridging score: was evaluated by 2 independent observers. The scale used: completely un-bridged (0% bridged); 0% - 50% bridged (as in some bridging exists but does not extend around more than 50% of the circumference); 50% - 90% bridged (bridging extends around more than 50% of the circumference but does not extend around more than 90% of the entire circumference); fully bridged (90-100% bridged).

Torsion test: Following μCT scanning, the tibiae were tested in torsion according to previously published methods (Morgan et al. Bone vol 44; 2009) in order to quantify torsional strength and torsional rigidity. The contralateral tibiae from control (group A) were also tested in order to allow quantification of the regain in strength and rigidity.

RESULTS:
By 15 days, histology of control fractures (group A) demonstrated large callus composed of cartilage and periosteal intramembranous bone. Calluses of fractures of groups B, C&D were significantly smaller and showed advanced healing as demonstrated by trabecular bone replacing cartilaginous callus mostly intramembrally. μCT analysis showed reduction in TV and BV in all experimental groups, as compared with the control group (p<0.0001). The least BV and TV were demonstrated in group D, consistent with the histological observations. BV/TV and TMD did not differ among treatment groups, although TMD increased over time as TV and BV both decreased (p<0.02). Analysis of the repair process by evaluation of the cortical bridging score showed that most animals independent of treatment exhibited bridging of 0-50%.

By 30 days, histology of control fractures (group A) demonstrated still a large callus, most cartilage was replaced by trabecular bone, however, more extensive areas of fibrous tissue can be seen centrally. Advanced repair was demonstrated in fractures of groups B and C and full repair demonstrated by bridging of cortices in group D. μCT analysis showed that mineralized callus volume was smaller in group D as compared to the control group (p<0.0001). Groups C and D exhibited the largest number of specimens with advanced cortical bridging (>50%). In comparison, bridging in all control specimens was <50%.

No differences in mechanical properties were found among groups, although the fractured tibiae in Group B exhibited higher torsional rigidity than that of the contralateral bones from group A (p=0.03).

DISCUSSION:
A model of transverse fractures in rat tibia was used for exploring the potential of local application of VEGF to enhance fracture healing. In addition, the adjunctive effect of VEGF to the enhanced repair by the combination of PTH1-34+IL-6+IL-6sR (that has been already shown by us) was tested.

Following a closed transverse fracture, the callus formed consisting of cartilage and periosteal membranous bone, gradually remodels into trabecular bone. This process takes about 6 weeks in the rat. Advancement of this regulated repair process is expressed by reduction of callus volume. Consistently, enhancement of the repair process, 15 days post fracture, was obviously demonstrated by reduction of BV and TV following application of VEGF compared to control (saline injected). When VEGF was combined with PTH1-34 and IL-6+IL-6sR its effect was even more pronounced as demonstrated by histology and μCT analyses. As bridging of the periosteal callus is hard to quantify with μCT but would likely affect the mechanical properties, we introduced a bridging score. By 15 days post fracture no differences between groups were notified (all specimens demonstrated 0-50% bridging score). However, 30 days post fracture, advanced cortical bridging (>50%) was shown in most specimens of groups C and D eventually predicting better mechanical properties by the enhanced healing. These observations were strengthened by the histological and μCT analyses. Histology demonstrated advanced repair of the fracture in groups B and C and full repair in group D. μCT analysis showed that mineralized callus volume was smaller in group D as compared to the control group (p<0.0001).

We conclude that advanced healing that was brought about by PTH1-34 and IL-6+IL-6sR was even more pronounced by the concomitant application of VEGF.