Low dose erythropoietin stimulates fracture healing in small segmental defects in mice

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INTRODUCTION:
Despite our growing knowledge on the mechanisms of fracture healing, about 5-10% of all fractures still show delayed healing or fail to heal (Marsh, 1998). Beyond its classical role in regulation of erythropoiesis, erythropoietin (EPO) has been shown to exert protective and regenerative actions in a variety of non-hematopoietic tissues. In a previous study we could show that daily application of 5000 U/kg EPO for 5 days (high-dose, short-time) enhances early endochondral ossification and mechanical strength in a closed femoral fracture model in mice (Holstein et al., 2007). However, the initial effect of short-time and high-dose application was found vanished after 5 weeks, probably because of the good healing response of mice in the closed fracture model and limitation of EPO administration to 5 days post fracture. Therefore, the aim of the present study is to analyze the impact of low dose and long-time EPO treatment on fracture healing in segmental defects in mice.

METHODS:
Right femora of CD-1 mice were prestabilized using a pin-clip technique, as described previously (Garcia et al., 2008). Briefly, an intramedullar pin was implanted retrograde in the right femur. Then, the femur was exposed through a lateral approach and a metallic clip was implanted ventro-dorsally through the medullary cavity, passing the intramedullar pin laterally. The operative procedure was performed using an operating microscope. Afterwards an osteotomy with a gap size of 0.25mm was created with a giggle wire saw in the middle of the femur, under the metallic clip.

16 CD-1 mice were treated daily with EPO (500U/kg body weight (bw)) by intraperitoneal injection. Additional 16 mice served as controls, which were treated by daily intraperitoneal injection of vehicle only. Animals were killed after 2 and 5 weeks by cervical dislocation. Fracture healing was evaluated by biomechanical (non-destructive 4-point bending) and histomorphometric analysis. Erythrocytes, hemoglobin and hematocrit were measured in whole blood samples at the end of the observation period. Additional animals were killed after 2 weeks for Western blot analysis (n=4 each group) and for analysis of circulating endothelial progenitor cells (VEGFR2+/Sca1+) by flow cytometry (n=6 each group). All experiments were performed in adherence to the National Institute of Health guidelines for the use of experimental animals and were approved by the German legislation on the protection of animals.

RESULTS:
Erythrocyte and hemoglobin concentration, as well as hematocrit were significantly greater in whole blood samples from EPO treated animals when compared to controls after 2 and 5 weeks. Radiological callus diameter was significantly greater in EPO-treated animals after 2 weeks. Radiological callus diameter also was significantly greater in EPO-treated animals after 2 and after 5 weeks compared to controls. Histomorphometric analysis showed no differences in periosteal callus diameter after 2 and after 5 weeks. However, EPO-treated animals showed significantly more bone, and significantly less cartilage and fibrous tissue in the periosteal callus area after 2 weeks when compared to controls. After 5 weeks, this difference was found vanished and periosteal callus in both groups consisted mainly of bone. After 2 weeks, 2/8 EPO-treated animals showed bone bridging of both cortices, compared to 0/8 in controls. After 5 weeks all EPO-treated animals (8/8) showed complete bone bridging compared to 4/8 control animals (p<0.05). After 2 and after 5 weeks, bending stiffness was significantly greater in EPO-treated animals compared to controls. Western blot analysis of PCNA expression, as an indicator of cell proliferation in the fracture callus, showed no difference between both groups. However, EPO-treated animals showed a significantly decreased protein expression of NfκB. Flow cytometry showed a significant increase in circulating EPC’s (VEGFR2+/Sca1+) in EPO-treated animals after 2 weeks compared to controls.

DISCUSSION:
We have shown that low-dose EPO treatment significantly improves healing of small segmental defects in mice. This is indicated by a better biomechanical stiffness of the healed femora and an increased radiological density of the callus after 2 and after 5 weeks. The increased, radiological density is in line with histological findings which showed a greater proportion of bone in the healing callus. These findings indicate that EPO does not quantitatively stimulate callus formation, but rather improves the quality of the callus with a significantly increased proportion of bone.

We have shown increased numbers endothelial progenitors cells in peripheral blood of EPO treated animals, which could be the reason for improved fracture healing in EPO treated animals. This finding is in line with previous studies which have shown that EPO is a potent stimulus for stem cell mobilization from the bone marrow (Heeschen et al., 2003). Mobilization of EPC during fracture has been shown to occur to some extend physiologically (Lee et al., 2008). Matsumoto et al. further have shown that circulating EPC are recruited to the fracture side and that bone healing is enhanced biomechanically, radiologically and histologically after intravenous transplantation of progenitor cells (Matsumoto et al., 2008).

In the future, EPO might help to stimulate fracture healing, especially in patients at high risk for non-union formation. Because, systemically administered EPO already is a widely used drug with a well known safety profile, translation into the clinic will be eased. Beyond its potential impact on the process of fracture healing, EPO has been shown to posses’ protective and regenerative properties in various in vivo models of tissue injury like the brain, after subarachnoidal bleeding, after spinal cord and peripheral nerve injuries, ischemia/reperfusion injury in the kidney and the heart. These tissue protective and regenerative properties of EPO are of special interest in trauma patients, who frequently suffer from injuries of multiple body compartments.

REFERENCES: