**Impaired Fracture Healing In Nrf2-Knockout Mice**

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**INTRODUCTION:** Detoxification of reactive oxygen species (ROS) and xenobiotics has been identified as a key mechanism of protection for physiologic cellular turnover. Almost any type of tissue can be seriously harmed by endogenous toxins like ROS that cause DNA breakage. After fracture of long bones a complex cascade of pathophysiological reactions follows the event of discontinuation of the weight bearing skeleton. The bony injury is accompanied by disruption of blood vessels and soft tissue leading to tissue ischemia and acidosis. Damaged blood vessels can not supply regeneratory cells with nutrients and oxygen. Within the wound a blood clot is formed, that is covered by platelets and integrated into a fibrin mesh that provides a static base for migrating macrophages, leukocytes and mast cells. Subsequently regeneratory cells (endothelial cells, smooth muscle cells and pluripotent mesenchymal stem cells) initiate bone repair by forming granulation tissue and differentiation into osteoblasts, fibroblasts and chondroblasts. Toxic metabolites like ROS are produced immediately after the damaging event causing lipid peroxidation that results in the production of malondialdehyde (MDA) and cell death via DNA breakage. In case of injuries to other tissues local or whole body oxidative stress is induced(1). In addition a number of studies have demonstrated the importance of physiologic antioxidants for the prevention of fractures. Despite these events in most cases fracture healing occurs successfully. The protecting mechanisms that prevent disturbances in fracture healing especially by toxic ROS have so far not been fully elucidated.

To counteract damage by ROS higher animals have developed a complex defence system consisting of detoxification enzymes and antioxidant proteins. In this system the transcription factor nuclear factor, erythroid-derived 2, like 2 (NF-E2-related factor 2, Nrf2) has been identified as a key molecule that controls expression of many antioxidant and detoxifying enzymes(2). The promoter region of so called “phase II” enzymes and other detoxifying enzymes carry the Antioxidant response element (ARE), a cis-acting enhancer element in their promoter region that binds Nrf2, a member of the Cap’n’Collar family of transcription factors. This Nrf2-ARE pathway acts as a master regulator of cellular protection. In brain injury Nrf2-deficient mice showed increased intestinal inflammatory response and enhanced expression of Nrf2-driven genes is believed to be neuroprotective. In recent studies cytoprotective effects in cardiac disease, atherosclerosis, lung emphysema, toxic liver damage, renal oxidative stress and preeclampsia(3) have been attributed to the Nrf2-ARE mediated cellular defense mechanism. With respect to fracture repair that involves callus formation as a critical step towards solid bony consolidation upregulation of Nrf2 was demonstrated in multipotent mesenchymal stem cells (MSC) after adrenaline exposure. Thereby the vulnerability of chondrogenic and osteogenic precursor cells to ROS could be reduced. Because oxidative stress can disturb the complex pathophysiological changes after skeletal injury we hypothesized that Nrf2 is essential for fracture healing. A standard femur fracture model was used to evaluate callus formation of the Nrf2 knockout mouse by µCT, histomorphometry and immunohistochemistry and a transgenic mouse model was used to demonstrate in vivo ARE-activation.

**METHODS:** We investigated femora from male 4 months old Nrf2/-/+ (control) and Nrf2/-/ mice at 14 and 28 days after fracture. Mice were obtained from our breeding colony after approval by our institutional animal studies committee. A closed mid-diaphyseal transverse fracture was created in the right femur by three-point bending after insertion of a rod into the medullary canal as described by Bonnarens. Femora were assessed after 2 and 4 weeks with respect to the callus morphology by x-ray, µCT and DXA(dual energy X-ray absorptiometry). Bones were investigated by calcified and decalcified histology and by microradiography. Immunohistochemical staining for collagen 1 and collagen 2 were performed. For detection of ARE-activity during fracture healing fractures were produced in the transgenic ARE-Luc mouse. In-vivo measurement of luminescence was recorded by a Xenogen Imager.

**RESULTS:** After 4 weeks callus size was 26.8±5.3 3.56 mm3 and 14.2 ±4.66mm3 (p=0.017) for wild-type and knock-out mice, respectively (Figure 2). Bone Mineral Density (BMD) and Whole body Composition (BMC) did not vary significantly. The histologic evaluation showed reduced callus formation and reduced mineralization in von Kossa staining. Microradiography revealed decreased trabecular density of knockout mice. Biomechanical testing showed decreased torsional rigidity in knockout mice.

Luciminscense recording demonstrated markedly increased activity of ARE in the fractured leg as opposed to the control side in the ARE-Luc mouse model. (Figure 2; arrow)

**DISCUSSION:** Fracture healing is a complex process that is poorly understood so far. Every surgeon well remembers cases of patients with impaired bone healing- a lot of them ultimately lose a limb after a long period of surgical and conservative attempts to treat the disease. The clinical scenario calls for specific treatment options i.e. the application of osteoinductive and osteoconductive substances. With respect to such therapeutic intentions the need for more detailed knowledge of the molecular regulating mechanisms becomes most evident. Many studies have dealt with predisposing factors for impaired bone development revealing several gene defects as for Osteogenesis imperfecta. In contrast there are few reports on genetic mechanisms of impaired bone healing. Our study adds one genetic defect that leads to impaired callus formation. When fractures occur the pathophysilogic response is characterized by a complex interaction of cells, platelets and signalling molecules that initiate and maintain bone healing. Nrf2 is a key molecule that regulates the so called “electrophilic counterattack response”. We demonstrate that the Nrf2-ARE pathway is activated in bone healing and therefore oxidative stress seems important in bone remodelling. Further studies will elucidate in how far induction of Nrf2 expression can improve bone healing by selectively activating the ARE-response Element.

**References**

