Oxidant status increased during fracture healing in rats

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We evaluated oxidant status during bone healing in 50 rats. In 40 rats, the right tibia was fractured and fixed intramedullarlyy (study leg) and the left tibia was pinned but not fractured (control leg). Rats were killed on days 1, 3, 7, 14, 28 and malondialdehyde (MDA) levels were determined in tibial bone tissue. The MDA levels of study and control legs were compared with basal MDA levels in bone in 10 rats. There was no apparent difference between the basal level and control legs, but the study legs showed a statistically significant increase in MDA levels on days 7 and 14. We conclude that no oxidative stress injury occurs during the ischemic period of fracture healing, but it may be significant during inflammation and the formation of callus.

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Partially reduced forms of oxygen, commonly known as oxygen-free radicals, are known to cause electrical and subcellular abnormalities in the tissues (McCord 1985). They have emerged as a major final common pathway of tissue injury in a wide variety of otherwise disparate disease processes (Parks et al. 1982, Farber et al. 1990, Van Ye et al. 1993). It has been shown that oxygen-free radicals are intermediaries in the formation and activation of osteoclasts (Garret et al.1990). In their experimental study, Göktürk et al. (1995) have demonstrated that increases in oxygen-free radicals impair fracture healing. A frequent cellular target of free radical attack is the lipid component of membranes, resulting in lipid peroxidation (Sugino et al. 1987, Hall 1993). To evaluate lipid peroxide content, levels of many intermediate products and end-products are used. The most reliable indicators are MDA or thiobarbituric acid reactive substance (TBA-RS) (Sugino et al. 1987, Oda et al. 1992). In this study, we investigated whether oxidant stress increased during the fracture healing process of rat tibias.

Animals and methods

50 male Sprague-Dawley rats with a median weight of 225 (218–235) g were used. Animals were anesthetized with ether, and fracture and/or fixation were performed. The fracture and fixation technique have been described in detail by Nordsletten et. al. (1994). The animals were operated on under sterile surgical conditions. The right lower leg was shaved and a longitudinal incision was made parallel to the anterior margin of the tibia. An 18 G cannula was inserted through the patellar ligament into the medullary canal. 4 mm distal to the tibial tuberosity, the anterior cortex was sectioned with a scalpel and the fracture completed by manual breaking of the posterior cortex leaving the fibula intact. The cannula was advanced distal to the fracture until it jammed in the medullary canal. 2 cannulae, 21G and 25G, were inserted inside the first and both were advanced past each other until jamming occurred in the distal fragment. The cannulae were cut flush with the bone at the insertion site and the wound was closed in two layers (study leg). Cannulae were inserted into the left tibias which had not been fractured (control leg). Postoperatively, each rat

was caged individually and allowed free access to water and a standard pellet diet. Light-dark cycle (14 L; 10 D) and temperature (24 °C) were controlled during the whole period of the experiment. The rats resumed weight-bearing within a few days and no infection developed.

To determine the basal MDA levels of bone, right and left tibial bone specimens were taken from 10 rats which had not been treated (control group). The remaining 40 rats, divided into groups of 8, were killed after being anesthetized with ether on days 1, 3, 7, 14, 28 and the intramedullary cannules were removed through a short incision. Bone from the right tibias was osteotomized to obtain specimens of 1 cm in length, including both callus and cortical bone. Equivalent specimens were taken from the left tibias. All specimens were washed with 0.9% NaCl to remove hematoma and then dried. They were stored in glass bottles separately and deep-frozen (–20 °C) pending biochemical analyses. The MDA method is based on measurement of the absorbance of thiobarbituric acid-malondialdehyde (Van Ye et al. 1993). Results are expressed in picomoles per milligram protein. This experimental study was reviewed and approved by the local ethics committee for animal research.

Statistics

One-way analysis of variance (ANOVA) was used to determine statistical significance among groups, and the paired t-test was used to detect a significant difference between the study and control legs. Statistical significance was set at a level of $p < 0.05$.

Results

MDA levels in the control legs (pinned but not fractured) did not differ significantly from basal levels (Table). The study legs (fractured and pinned) showed no differences on postfracture days 1 and 3, but MDA levels were significantly higher on days 7 and 14 than basal bone MDA levels ($p < 0.01$) (Table). At 28 days after fracture, the levels of MDA in fractured bones were not significantly different from that of the basal level. When we compared the values of study and control legs, MDA levels were increased in study legs on postfracture days 7 and 14 ($p = 0.01$).

Bone malondialdehyde (MDA) levels in 3 groups, mean (SD)

Days	Control group n 10	Control leg n 40	Study leg n 40
0	1.85 (0.42)		
1		1.97 (0.51)	1.73 (0.42)
3		1.77 (0.38)	1.73 (0.33)
7		1.73 (0.78)	2.50 (0.66)
14		1.66 (0.64)	2.69 (0.88)
28		1.67 (0.70)	2.07 (0.59)

Discussion

In one study, a tonometer system was used to measure the PO_2 in healing bone defects in the rib of dogs (Heppenstall et al. 1975). Oxygen tensions were 8 mm Hg at 3 days, 32 mm Hg at 3 weeks, and 46 mm Hg at 6 weeks after the defect. The arterial PO_2 was 102 mm Hg. The authors concluded that the low PO_2 found in a healing bone defect was due not to an increase in O_2 consumption but to a decrease in delivery of O_2 to the fracture site. In addition, it has been shown that a fracture hematoma includes excess hyaluronic acid in the early period of fracture healing (Hult 1989). Hyaluronic acid has antioxidant effect, and begins to decrease 7 or 8 days after a fracture. All these mechanisms explain why MDA levels are not higher but slightly lower during the first days of fracture healing.

In oxidative events causing organ and tissue injury, stress injury is caused by an ischemia-reperfusion mechanism (Ikeda and Long 1990, Oda et al. 1992, Rangan and Bulkley 1993). Reduced blood flow to a fracture results in regional ischemic injury, and in the peripheral regions of the "ischemic zone", there are viable cells that can be salvaged, if managed adequately. Otherwise, these cells undergo irreversible injury. This phenomenon is referred to as reperfusion injury and may reduce bone healing. In many studies examining the relationship between myocardial injury and lipid peroxidation, it has been shown that oxygen-free radicals are unchanged or decreased during

the ischemic period, but increased significantly in the reperfusion period (Dhaliwal et al. 1991, Kirshenbaum et al.1993). Similarly the first 3 days of fracture healing may be compared to the ischemic period. Thus during this period, no oxidative stress injury occurs. After this, in the stage of callus formation, in addition to fibroblast and collagen cells, new capillary vessels with other inflammmatory cells increase the production of oxygen-free radicals which may cause oxidative injury to fractured bones, as seen in the other tissues with reperfusion injury (Cornell and Lane 1992). In our study, MDA levels rose significantly in the period of callus formation. This increase in the second and third weeks is probably caused by increased vascularization and oxygenation which may be a reaction to the previous relative ischemia. We believe PNL, which is one of the main cellular agents in tissue regeneration and repair, may play a major role in the development of oxidative stress injury. A significant increase in MDA during this period is comparable to the ischemia-reperfusion injury seen in other types of organ and tissue trauma (Ikeda et al. 1989, Bagchi et al. 1990, Oda et al. 1992, Rangan and Bulkley 1993). Although MDA levels 4 weeks after the fracture were slightly above basal levels, this increase was not statistically significant. After 3 weeks, cartilage callus begins to become bony callus, and cellular oxidation in the region of the fracture declines.

Intramedullary pinning is an acceptable method for treating fractures, but may disturb the medullary blood supply. In this study, opposite tibias were pinned without fracture to examine the effect of pinning and fracture on MDA generation separately. This study showed that intramedullary pinning of tibias did not increase MDA levels. This enabled us to assume that in the study group, elevation of the rise in MDA levels was mainly due to the fracture, and not the pinning.

In conclusion, our findings show that oxidative stress injury in a fracture begins only after the avascular and anoxic periods are over. With increased vascularization, severe oxidation occurs and lasts until callus formation. As the cellular and subcellular changes in reperfusion injury have been shown to be improved by different pharmacological agents (Kirshenbaum et al. 1993), we recommend that their effects should be studied further in fracture healing because oxidants increases only in the second and third weeks of fracture healing.

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