Oxygen-free radicals impair fracture healing in rats

Erol Göktürk[1], Akin Turgut[1], Cengiz Bayçu[2], Izge Günal[1], Sinan Seber[1] and Zafer Gülbas[3]

We studied the effect of oxygen-free radicals on fracture healing. 30 male rats were divided into 2 groups: 15 rats were given saline 5 mL/kg i.p. (control group) and 15 were given zymosan 100 mg/kg i.p. to induce oxygen-free radicals through stimulation of NADPH oxidase in polymorphonuclear leucocytes. 1 hour later, the right forelimbs of the rats were broken by light manual compression. These treatments were given once a day until the fifth postfracture day. All rats were killed on day 22, and histological sections of the radius and ulna were examined without knowledge of the treatment given. The administration of zymosan impaired the fracture healing and therefore oxygen-free radicals appear to play an important role in fracture healing.

Department of [1]Orthopaedics and Traumatology, [2]Histology and Embryology and [3]Hematology, University of Osmangazi, Faculty of Medicine, 26480 Eskisehir, Turkey. Tel +90 22223-92979. Fax -91477
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During the past decade, considerable attention has been devoted to the deleterious effects of oxygen-free radicals in many pathological processes (McCord 1985, Ikeda and Long 1990). Ischemia-perfusion (Parks et al. 1982, Korthuis et al. 1985, Bulkley 1983, 1987), administration of xenobiotics (Nishikimi et al. 1972, Maridonneau-Parini et al. 1986), or bacteria (Babior 1978) stimulate epithelial or inflammatory cells to produce oxygen-free radicals in many tissues. Garrett et al. (1990) demonstrated that oxygen-free radicals are intermediaries in the formation and activation of osteoclasts.

The mechanisms of oxygen-free radical generation are well known. However, to our knowledge, no published studies have investigated whether production of these compounds affects the healing of fractures. We investigated the effect of oxygen-free radicals on fracture healing in rats.

Animals and methods

30 male Sprague-Dawley rats, weighing 200–300 g, were used. The rats were divided randomly into control and zymosan groups (15 rats each). The mean weights of the control group and of the zymosan group were 248 g and 233 g, respectively. 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 were anesthetized with ether and their right forelimbs were then broken by light manual compression (Allen et al. 1980). Immediately after fracture while still under anesthesia, the rats were radiographed to ensure that each rat had a uniform, simple transverse fracture of the radius and ulna.

1 hour before the fracture the following treatments were given to the 2 groups: either saline 5 mL/kg i.p. once a day (control group), or zymosan 100 mg/kg i.p. (Sigma Chemical Co.) once a day, to induce oxygen-free radicals by stimulation of NADPH oxidase in polymorphonuclear leucocytes (Foschi et al. 1988, 1990). Treatments were stopped on the fifth postfracture day.

All rats were weighed daily, and the dose adjusted accordingly. Zymosan was prepared daily and all rats were given proportional amounts of saline.

The rats underwent the treatment without complications. There were no differences between the final body weights. At the end of the study, the mean weight of the control group was 330 g, and of the zymosan group 325 g.

At 7, 14, and 21 days postfracture, the rats in the both groups were anesthetized with ether and the fractures were radiographed. The serial radiographs were used to determine the proper time to terminate the study. When fractures in the control rats showed radiographic evidence of mineralized bridging of fracture sites, the animals were killed with high doses of ether and autopsied (day 22). Autopsy was limited to removal of the fractured forelimbs by severing the carpus and cubitus. The tissue was fixed in 10% neutral buffered formalin. The forelimb bones were

Table 1. Fracture healing scores. Grading system

Histologic evaluation	Grade
Pseudoarthrosis formation	0
Incomplete cartilaginous union	1
Complete cartilaginous union	2
Incomplete bony union	3
Complete bony union	4

decalcified with 15% aqueous formic acid. The radius and ulna were separated and embedded in paraffin to allow for 6 µm sections through the center of each callus (Allen et al. 1980). All the histologic sections were stained with hematoxylin and eosin for light microscopic examination. The extent of healing of each fracture was determined after study of the entire serial sequence, approximately 50 sections. The histologic grading of healing was based on the numerical 5-grade system (0–4) which was described in detail by Allen et al. (1980) (Table 1). The least healed bone (radius or ulna) dictated the grade. However, when a decision was equivocal, due to irregularities of sectioning, the degree was determined by examining both fracture callus sections (Allen et al. 1980). The grading was done without knowledge of which treatment had been given.

The chi-square test and t-test were used for comparison between groups. A value of $p < 0.05$ was considered significant.

Results

All fractures in the control group showed radiographic evidence of healing at the 21st postfracture day. Histopathologically, all fractures in the control group were found to have an incomplete bony union (grade 3), whereas union in the zymosan-treated group was either by incomplete bony union (n 9) or complete cartilaginous union (grade 2, n 6, $p < 0.01$).

Discussion

Fracture healing appears to proceed through 3 distinct stages: 1) inflammatory stage, 2) callus formation stage, and 3) remodeling stage (Simmons 1985). The inflammatory stage, which involves the first 5 days after fracture, and is characterized by formation of the fracture hematoma, provides the modulation and induction of cells needed for the repair process. Inflammation begins within 48 hours and lasts until cartilage and bone appear. The first cells to arrive at the fracture site are inflammatory cells consisting of polymorphonuclear leucocytes, macrophages, and mast cells (Simmons 1985, Cornell and Lane 1992). In this period, osteoclasts also appear and begin to remove necrotic bone. Fibroblasts and invading capillaries next appear. Fracture hematoma is very rapidly replaced by granulation tissue consisting of inflammatory cells, fibroblasts, collagen and invading capillaries (Cornell and Lane 1992).

The early stage of fracture healing is very important. The causes of most biologic failures act within the first weeks after the fractures (Frost 1989).

Oxygen-free radicals, generated through polymorphonuclear leucocytes (PMN) activation (Babior et al. 1976, Tauber et al. 1979), are known to impair wound healing and granulation tissue (Foschi et al. 1988, 1990). In their investigations of the effect of oxygen-free radicals on wound healing and granulation tissue, Foschi et al. (1988, 1990) used zymosan to induce oxygen-free radicals by stimulation of NADPH oxidase in PMN cells. They used zymosan 100 mg/kg for 5 days and showed that this dosage is suitable for producing oxygen-free radicals comparable to the amount found in pathological conditions.

When exposed to appropriate stimuli, PMN undergoes a series of metabolic changes, collectively termed the respiratory burst. The respiratory burst of PMN produces several oxygen-free radicals (Babior et al. 1976, 1978, Tauber et al. 1979). Zymosan can activate the respiratory burst (Goldstein et al. 1975, Tauber et al. 1979, Cohen et al. 1980, Maridonneau-Parini et al. 1986).

Zymosan, a 3–5 µm cell wall fragment of Saccharomyces cerevisiae, has been analyzed and found to contain a mixture of polysaccharides, protein and lipid (DiCarlo and Fiore 1957, Fitzpatrick and DiCarlo 1964). Glucan, a ß-linked branched-chain polysaccharide of glucose subunits, which constitutes the inner portion of the wall, forms up to 60% of the dry weight of zymosan. A second polysaccharide, mannan, composed of α-linked mannose subunits, constitutes another 20% (Williams et al. 1986). Although the major component of zymosan responsible for PMN-activation is glucan, the mannan component of zymosan also plays an important role in the transmembrane activation of the respiratory burst in these cells (Williams et al. 1986).

According to the above evidence, oxygen-free radicals would be expected to impair fracture healing. However, to our knowledge, this point has not been studied. We used zymosan for 5 days during the inflammatory stage of fracture healing and found that it impaired treatment. Our findings suggest oxygen-free radicals may play a role in fracture healing.

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