Lipopolysaccharide impairs fracture healing
An experimental study in rats

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Background  It has been shown that trauma causes translocation of lipopolysaccharide (LPS) endotoxins from the gut. LPS has been identified as a major bacterial bone resorbing factor. The effects of LPS on bone healing are therefore of clinical interest, as trauma involving fractures followed by sepsis is a clinical scenario. We investigated the effects of systemic and local administration of LPS on the healing of femoral fractures in rats.

Animals and methods  In 3 groups, each consisting of 9 rats, a mid-diaphyseal osteotomy/fracture of the femoral bone was performed and then nailed. In one group of animals, LPS was applied intraperitoneally (systemically), and in another group, LPS was applied locally at the fracture site. The third group served as a control. The animals were killed after 6 weeks, and the mechanical characteristics of the healing osteotomies were evaluated.

Results  We found that LPS induced a hypertrophic and immature callus, as evaluated by bone mineral content and density. In the rats given LPS intraperitoneally, the mechanical strength characteristics were reduced, as evaluated by bending moment, rigidity, and energy absorption.

Interpretation  The rats given LPS intraperitoneally reflect a clinical situation with fracture trauma and endotoxinemia. Our findings indicate that endotoxinemia may impair the fracture healing processes.

The initial phase of tissue injury is characterized by inflammation and the formation of fibrin clots.
Animals and methods

27 male Wistar rats (Møllegårds Avslaboratorium, Eiby, Denmark) weighing 352–379 g were used. Following subcutaneous anesthesia (hypnorm/dormicum), the left femur was exposed between the lateral vastus and the hamstrings. The muscles were carefully elevated in the lateral part. We performed a partial, transverse osteotomy at the midshaft of the bone 12 mm from the top of the greater trochanter with a fine-toothed circular saw blade mounted on an electrical drill, followed by manual breakage. The medullary canal was successively reamed from the osteotomy site in proximal and distal direction to a diameter of 1.6 mm, using steel burrs mounted on the electrical drill. The fractures were reduced, and a 1.6-mm steel pin was inserted from the trochanter area for stabilization. Fixation was achieved without radiographic controls. Proper pin placement was confirmed later from radiographs taken at the end of the experiment. The wounds were closed in two layers.

The rats were allocated to 3 groups: the local group, the intraperitoneal group and control group. The local group underwent injection of 0.2 mL of 1 mg/mL LPS solution (i.e. 200 µg/animal) at the fracture site and injection of 0.2 mL isotonic saline intraperitoneally each day over 7 days, with start at fracture day. The intraperitoneal group underwent injection of 0.2 mL of 1mg/mL LPS solution (i.e. 200 µg/animal) intraperitoneally (systemically) and 0.2 mL saline at the fracture site over 7 days. The control group underwent injection of 0.2 mL saline at the fracture site and intraperitoneally over 7 days.

6 weeks after fracture, the rats were killed and the fractured left femur was dissected free from all soft tissue. Anteroposterior and transverse diameters of the callus area were measured with a sliding calliper (accuracy 0.01mm). The quantity of the callus was expressed as the cross-sectional area, assuming it to be an ellipse.

The bones were examined radiographically, and the intramedullary pin was removed. The left femur was examined by dual-energy X-ray absorptiometry (DEXA). DEXA was performed on a GE Lunar Piximus (Lunar Corporation, Madison, WI) supplied with a tube current of 400 µA. The frequency of the scanning unit was 50–60 Hz, and the X-ray tube had a focal spot size of 0.25 mm × 0.25 mm with a coefficient of variation of 1%. Each femur was placed on a water suspension during scanning. After the scanning of the total femur, a 3-mm region of interest (ROI) was measured, centralized 12 mm from the top of the greater trochanter (corresponding to the callus region in the left femur diaphysis).

The healing fractures were tested by cantilever bending. The proximal end was fixed with a clamp, the cam of a rotating wheel engaged the femoral condyles, and a fulcrum at the osteotomy site was the third point of application of force. Refracture, then, was performed by deflection of the distal half of the femur, as previously described (Engesaether et al. 1978). The testing machine was run at a constant rate of 0.08 rad/sec. The load values were transformed to a chart recorder displaying the load deformation curve. The strength was calculated as the bending moment necessary to produce refracture. The bending rigidity was determined from the slope of the linear elastic part of the curve. The fracture energy was defined as the energy absorbed during loading to refracture.

Data are presented as mean and standard error of the mean. To test statistical differences, ANOVA was used followed by LSD test where appropriate. The level of significance was set at 0.05.

Results

4 rats died during the first operative day. The remaining rats tolerated the operation well and resumed full weight bearing after a few days. There were no failures, and all fractures healed by production of external callus, as defined by radiography. The animals were weighed at operation and at sacrifice. There were no differences between the groups regarding weight at operation and weight measured 16 weeks after fracture.

The callus area was lower in the control rats than in rats given LPS intraperitoneally (p = 0.008) and locally (p = 0.02) (Table 1). Bone mineral density and bone mineral content of the callus area were both reduced in the rats given LPS, either intraperitoneally or locally, as compared to the control rats (intraperitoneal group vs. control group: BMD, p < 0.001; BMC, p < 0.001; local group vs. control...
Bending moment was reduced in the rats given LPS intraperitoneally as compared to the control group (p = 0.05) and to the rats given LPS locally (p = 0.006) (Table 2). The differences in rigidity did not reach statistical significance, but fracture energy was reduced in the rats given LPS intraperitoneally as compared to the rats given LPS locally (p = 0.01).

### Discussion

In this study, we investigated the effects of LPS in the initial inflammation phases after fracture—which may be most critical for healing. We found that both intraperitoneal and local application of LPS increased callus production. On the other hand, both bone mineral content and bone mineral density of the callus were reduced. These observations reflect a hypertrophic and immature callus in the rats given LPS relative to the control group. The consequences were impaired fracture healing of the rats given LPS intraperitoneally. In the rats given LPS locally, increased callus production appeared to compensate for the immaturity. LPS at a dose of 6 mg/kg causes multiple organ dysfunction and death in rats (Ruetten and Thiermann 1997). We therefore used one-tenth of this dose in order not to cause general illness in the rats given LPS. This was confirmed by the observation that all rats gained weight to the same degree during the 6-week experimental period.

Healing of a femoral fracture in rats takes about 12–14 weeks before normal strength has been regained (Ekeland et al. 1981). At 6 weeks, the gain in fracture strength is about 50% of that of intact bone. Effects of fracture interventions are detected most reliably in the early phases of bone regeneration, and our experiments were thus terminated after 6 weeks.

Although LPS has been identified to be a bone-resorbing factor (Nair et al. 1996), very little is known about its mechanism of action. Two pathways have been suggested in LPS-induced bone resorption in vivo (Chiang et al. 1999). At high doses, LPS-induced bone resorption is mediated at least in part by IL1 and TNF receptor signaling. At low doses, however, LPS-induced bone resorption proceeds through a pathway independent of IL1 and TNF signaling. In our study, we used rather low doses of LPS to mimic a clinically relevant situation, and our observations support the assumption that LPS impairs bone metabolism. This is the first report to show that this has consequences for normal fracture healing.

Fracture healing requires the induction of mesenchymal stromal cells to differentiate along the osteoblast lineage for new bone formation. Mesenchymal stem cell recruitment, as well as proliferation and differentiation, are regulated by growth factors. The macrophages have a significant role in expression of inflammatory cytokines and growth factors (Danon et al. 1998). Thus, it has been hypothesized that the macrophage cell lines have a central role in wound and fracture healing (Champagne et al. 2002). However, the induction of inflammatory cytokines and fibrosis-promoting growth factors by stimulated macrophages suggests a prominent negative or osteolytic effect (Kovacs and DiPietro 1994). Under proinflammatory conditions such as exposure to LPS, the macrophage loses its ability to synthesize BMP-2. On this
basis, it has been suggested that osseous healing is inhibited by conditions that promote proinflammatory activity of the macrophage (Champagne et al. 2002). This suggestion is supported by our study.

In contrast to local administration, intraperitoneal administration of LPS is believed to enter the portal circulation. Hepatic macrophages (Kupffer cells) play a critical role in the immune response to injury. Kupffer cells constitute the largest fixed macrophage population of the body. As indicated in our study, it may be assumed that direct stimulation of this large population of hepatic macrophages gives stronger effects than stimulation of local and lesser populations of macrophages. Alternatively, LPS may induce a stronger proinflammatory response in Kupffer cells than in peripheral macrophages.

It has been found that femoral fracture rapidly causes ultrastructural changes of Kupffer cells characteristic of activation, reflecting early hepatic macrophage adaptation and conferring an anti-microbial and less proinflammatory phenotype (Huynh et al. 1997). It has been suggested that these functional alterations represent a response to humoral factors released by traumatized tissue or neuroendocrine effects.

In contrast to a peripheral trauma, endotoxinemia is believed to alter the general response towards a more proinflammatory state. In general, this may impair the well-balanced response required for efficient fracture healing. Endotoxinemia should be considered to be a frequent phenomenon in skeletal trauma, because the normal functioning of the gut barrier seems to be easily disturbed if the circumstances are suitable and the host is susceptible (Buttenschoen et al. 1996). Kupffer cells reside at a strategic position in hepatic sinuoids, interacting with mediators from the gut. These cells have thus been implicated in responses to LPS, and they play a pivotal role in defence in the event of trauma and endotoxinemia. In our study, the rats given LPS intraperitoneally reflect a clinical situation with fracture and endotoxinemia. Although this may not be a life-threatening situation, we have clearly demonstrated that endotoxinemia may influence the fracture healing processes.

No competing interests declared.
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