Lipopolysaccharide impairs fracture healing

An experimental study in rats

Olav Reikerås[1], Hamid Shegarfi[1], Jacob E Wang[2] and Stein E Utvåg[3]

[1]Department of Orthopaedics, [2]Institute of Surgical Research, Rikshospitalet University Clinic, [3]Akershus University Hospital, Institute of Clinical Medicine, University of Oslo, Norway
Correspondence OR: olav.reikeras@rikshospitalet.no
Submitted 05-02-03. Accepted 05-05-16

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. Affected tissue is invaded by hematopoietic cells, including neutrophils and peripheral monocytes that give rise to macrophages. These play a central role in wound healing, mainly by secreting cytokines involved in inflammation and by secreting reparative growth factors (Danon et al. 1998). Inflammation is a likely feature of bone repair processes, and several cytokines have been reported to be involved in bone metabolism (Girasole et al. 1994, Einhorn et al. 1995, Lader and Flanagan 1998, Ueda et al. 1998, Ohlin et al. 1999, Suda et al. 1999). Experimental and clinical experience indicates that trauma causes translocation of lipopolysaccharide (LPS) endotoxin from the gut (Hiki et al. 1995, Buttenschoen et al. 2000). LPS is a potent trigger of the mediator cascade and the inflammatory response (Tobia et al. 1997), and it has been identified as a major bacterial bone resorbing factor (Nair et al. 1996). The effects of LPS on bone metabolism are therefore relevant to clinical challenge of bone healing, as trauma involving fractures followed by endotoxinemia/sepsis is a clinical scenario. To our knowledge, however, no studies have been published which examine the effects of LPS on fracture healing. Local accumulation of LPS may have effects on the inflammatory response that are different from those of systemic accumulation. We investigated the effects of systemic and local administration of LPS on the healing of femoral fractures in rats.

Copyright© Taylor & Francis 2005. ISSN 1745–3674. Printed in Sweden – all rights reserved.
DOI 10.1080/17453670510045327

Animals and methods

27 male Wistar rats (Møllegårds Avlslaboratorium, 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

Table 1. Callus area (mm^2), bone mineral content (BMC, 10^{-3} g) and bone mineral density (BMD, 10-3 g/cm2) of the callus 6 weeks after femoral fracture in rats. Mean (SE)

	Control group	LPS intraperitoneally	LPS locally	P-value (ANOVA)
Callus area	50 (5.5)	70 (5.4)	66 (2.5)	0.02
BMD	203 (16)	124 (9.8)	156 (11)	0.001
BMC	59 (4.0)	35 (3.9)	47 (3.7)	0.001

Table 2. Bending moment (N), rigidity (N/mm) and energy (N mm) of the callus 6 weeks after femoral fracture in rats. Mean (SE)

	Control group	LPS intraperitoneally	LPS locally	P-value (ANOVA)
Bending moment	8.8 (2.1)	3.1 (0.5)	11 (2.5)	0.02
Rigidity	1.9 (0.3)	1.0 (0.2)	2.2 (0.5)	0.06
Energy	24 (7.2)	6.8 (2.0)	34 (10)	0.04

group: BMD, $p < 0.001$; BMC, $p = 0.04$) (Table 1).

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 antimicrobial 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 proinflammaory 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.

Buttenschoen K, Berger D, Hiki N, Strecjker W, Seidelmann M, Beger H G. Plasma concentrations of endotoxin and antiendotoxin antibodies in patients with multiple injuries: a prospective clincial study. Eur J Surg 1996; 162: 85-60.

Buttenschoen K, Fleisschmann W, Haupt U, Kinzl L, Buttenschoen D C. Translocation of endotoxin and acute-phase proteins in malleolar fractures. Trauma 2000; 48: 241-5.

Champagne C M, Takebe J, Offenbacher S, Cooper L F. Macrophage cell lines produce osteoinductive signals that include bone morphogenic protein-2. Bone 2002; 30: 26-31.

Chiang C Y, Kyritsis G, Graves D T, Amar S. Intereukin-1 and tumor nekrosis factor activities partially account for calvarial bone resorption induced by local injection of lipopolysaccharide. Infect Immun 1999, 67: 423-6.

Danon D, Kowatch M A, Roth G S. Promotion of wound repair in old mice by local injection of macrophages. Proc Natl Sci USA 1998; 86: 2018-20.

Einhorn T A, Majeska R J, Rush E B, Levine P M, Hororwitz M C. The expression of cytokine activity by fracture callus. J Bone Miner Res 1995; 10: 1272-81.

Ekeland A, Engesater L B, Langeland N. Mechanical properties of fractured and intact rat femora evaluated by bending, torsional and tensile tests. Acta Orthop Scand 1981; 52: 605-13.

Engesæther L B, Ekeland A, Langeland N. Methods for testing the mechanical properties of the rat femur. Acta Orthop Scand 1978; 49: 5125-8.

Girasole G, Passeri G, Jilka R L, Manolagas S C. Interleukin 11: a new cytokine critical for osteoclast development. J Clin Invest 1994; 93: 1516-24.

Hiki N, Berger D, Butenschoen K. Endotoxinemia and specific antibody behavior against different endotoxins following multiple injuries. J Trauma 1995; 38: 794-801.

Huynh T, Baker C C, Bracey L W, Lemasters J J. Adaptive Kupffer cell alterations after fracture trauma in rats. Am J Physiol 1997; 272: G1457-62.

Kovacs E J, DiPietro L A. Fibrinogenic cytokines and connective tissue production. FASEJ 1994; 8: 854-6.

Lader C S, Flanagan A M. Prostaglandin E2, interleukin 1 alpha, and tumor necrosis factor-alpha increase human osteoclast formation and boner resorption in vitro. Endocrinology 1998; 139: 3157-64

Nair S, Meghji P S, Wilson M, Reddi K, White P, Henderson B. Bacterially induced bone destruction: mechanisms and misconceptions. Infect Immun 1996; 64: 2371- 80.

Ohlin A, Sjogren U, Lerner U H. Bone resorbing activity released from zymosan-activated mouse peritoneal macrophages- the role of prostanoids and interleukin1. Inflamm Res 1999; 48: 181-92.

Ruetten H, Thiemermann C. Effect of calpain inhibitor I, an inhibitor of the proteolysis of I kappa B, on the circulatory failure and multiple organ dysfunction caused by endotoxin in the rat. Br J Pharmacol 1997; 121: 695-704.

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie M T, Martin T J. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 1999; 20: 345-57.

Tobia P S, Gegner J, Tapping R, Orr S, Mathison J, Lee J D, Kravchenko V, Han J, Ulevitch R J. Lipolpolysaccharide dependent cellular activation. J Periodontal Res 1997; 32: 99-103.

Ueda N, Koide M, Ohguchi M, Ishihara Y, Noguchi T, Okahashi N, Nishihara T. Involvement of prostagalndin E2 and Interleukin-1 alpha in the differentiation and survival of osteoclasts induced by lipopolysachharide from actinobacillus actinomycetemcomitans Y4. J Periodontal Res 1998; 33: 509-16.