Cumulative Effects of Bone and Soft Tissue Injury on Systemic Inflammation: A Pilot Study

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Abstract

Background In multiply injured patients, bilateral femur fractures invoke a substantial systemic inflammatory impact and remote organ dysfunction. However, it is unclear whether isolated bone or soft tissue injury contributes to the systemic inflammatory response and organ injury after fracture.

Questions/purposes We therefore asked whether the systemic inflammatory response and remote organ dysfunction are attributable to the bone fragment injection, adjacent soft tissue injury, or both.

Methods Male C57/BL6 mice (8–10 weeks old, 20–30 g) were assigned to four groups: bone fragment injection (BF, $n = 9$) group; soft tissue injury (STI, $n = 9$) group; BF + STI ($n = 9$) group, in which both insults were

applied; and control group, in which neither insult was applied. Animals were sacrificed at 6 hours. As surrogates for systemic inflammation, we measured serum IL-6, IL-10, osteopontin, and alanine aminotransferase (ALT) and nuclear factor (NF)- κ B and myeloperoxidase (MPO) in the lung.

Results The systemic inflammatory response (mean IL-6 level) was similar in the BF (61.8 pg/mL) and STI (67.9 pg/mL) groups. The combination (BF + STI) of both traumatic insults induced an increase in mean levels of inflammatory parameters (IL-6: 189.1 pg/mL) but not in MPO levels (1.21 ng/mL) as compared with the BF (0.82 ng/mL) and STI (1.26 ng/mL) groups. The model produced little evidence of remote organ inflammation.

Conclusions Our findings suggest both bone and soft tissue injury are required to induce systemic changes. The absence of remote organ inflammation suggests further fracture-associated factors, such as hemorrhage and fat liberation, may be more critical for induction of remote organ damage.

Clinical Relevance Both bone and soft tissue injuries contribute to the systemic inflammatory response.

Each author certifies that he or she, or a member of his or her immediate family, has no funding or commercial associations (eg, consultancies, stock ownership, equity interest, patent/licensing arrangements, etc) that might pose a conflict of interest in connection with the submitted article.

All ICMJE Conflict of Interest Forms for authors and *Clinical Orthopaedics and Related Research* editors and board members are on file with the publication and can be viewed on request. Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research. This work was performed at the University of Pittsburgh Medical Center, Pittsburgh, PA, USA

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Introduction

Long-bone fractures frequently occur in patients with polytrauma [3]. In particular, bilateral femur fractures result from high-velocity impacts, leading to complex fractures and severe injuries of the surrounding soft tissue envelope [35]. These injuries are characterized by high blood loss [6], severe generalized autodestructive inflammation [16], and remote organ damage [6, 16, 28].

Assessing the systemic inflammatory response can be useful adjuncts for clinically evaluating severely injured patients [11, 13]. For example, IL-6 levels have been

associated with the development of adverse events after multiple trauma [11]. Pape et al. [25], performing a prospective, randomized, multicenter study analyzing the inflammatory response (IL-6 and IL-1) after primary definitive treatment (< 24 hours) of femoral shaft fracture and damage control orthopaedic surgery, demonstrated the damage control strategy was associated with sustained inflammatory response. Furthermore, other investigations indicated delayed treatment would be advantageous in patients with IL-6 levels of more than 500 mg/mL [11].

Studies of cytokine pattern and the evolution of remote organ dysfunction after bilateral femur fractures in mice [13, 18, 21] demonstrate such trauma initiates a complex systemic inflammatory response in which the pro- and antiinflammatory cytokines, such as IL-6 and IL-10, play a pivotal role in systemic inflammation [18, 21]. Kobbe et al. [19] demonstrated fracture-associated soft tissue injury is a major contributor to the systemic inflammation found after long-bone fractures. IL-6 is more closely tied to soft tissue injury than other cytokines [29, 30], but both IL-6 and IL-10 correlate with the systemic inflammatory response and injury severity [1, 10, 24]. Locally exposed bone components to injured soft tissue induce a substantial release of pro- and antiinflammatory cytokines and acute lung injury [17]. Our study group recently published long-term (up to 72-hour) experiments using a mouse model [7] and showed peak IL-6 and IL-10 levels and increased remote organ inflammation within the liver and lung occurred 6 hours after trauma. However, it remains unclear whether the release of fragments of bone into the adjacent tissue per se contributes to systemic inflammation. Moreover, it is unclear whether fracture-associated bone injury and soft tissue act cumulatively or synergistically on the posttraumatic systemic inflammatory response and remote organ injury.

Pulmonary myeloperoxidase (MPO) activity is a marker of infiltration and accumulation of polymorphonucleocytes in lung tissue [12], demonstrating secondary organ and tissue damage. Various other markers of inflammation have been found in prior studies. Among these is osteopontin (OPN), a bioactive protein with a broad range of functions: inflammatory response, tissue and bone repair, wound healing, and tumor genesis [8, 34]. Its role as a chemotactic agent and activator of neutrophils and macrophages has been previously described [20, 23]. The function of OPN during posttraumatic inflammation is not fully understood.

In this preliminary study, using a mouse model, we addressed the following questions: (1) Does isolated fracture-associated bone liberation contribute to the systemic inflammatory response? And (2) does fracture-associated soft tissue injury in combination with the exposure of bone components affect the posttraumatic inflammatory response and remote organ inflammation?

Materials and Methods

We divided 33 mice into the following four groups: control group ($n = 6$); bone component injection alone (BF) group ($n = 9$); soft tissue injury alone (STI) group ($n = 9$); and combined bone component injection and soft tissue injury (BF + STI) group ($n = 9$) (Fig. 1). The end point in all experiments was 6 hours as per the peak in our previous study [7]. Mice were housed in accordance with NIH animal care guidelines. We used male C57/BL6 mice (Charles Rivers Laboratories Inc, Wilmington, MA, USA; 6–10 weeks old; weighing 20–30 g) in this study. Animals were maintained in the animal research center with a 12-hour-12-hour light-dark cycle and free access to laboratory feed and water. Pentobarbital (70 mg/kg intraperitoneal) was used for animal anesthesia. Isoflurane (Abbott Laboratories, Chicago, IL, USA) was administered to provide additional anesthesia. Buprenex (Bedford Laboratories, Bedford, OH, USA) (0.5 mg/kg) was injected subcutaneously before the induction of trauma. The research protocol for this study was approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh (Protocol Number 0801799).

The bone components for injection were obtained as follows. Six donor mice (one donor for three recipient mice) were euthanized via an overdose administration of pentobarbital. Femurs and tibiae were dissected and disarticulated. Bones were pulverized with a sterile mortar and pestle in a biohazard hood for 3 to 5 minutes, suspended with 2 mL phosphate buffer solution (PBS), crushed again, and kept in a sterile tube. In the BF group, the recipient mice were anesthetized, and 0.15 mL bone mixture suspended in PBS was injected using a 1-mL syringe and an 18-gauge needle in each thigh. In the STI group, the mice were anesthetized, and the soft tissue around each thigh was clamped with a hemostat for 30 seconds simulating a soft tissue injury. At the end of the experiment, a necropsy was performed to prove standard size and severity of the soft tissue injury. Moreover, we excluded the presence of severe local hematoma that may affect local and systemic inflammatory response. In the BF + STI group, the mice were anesthetized, the soft tissue around each thigh was clamped with a hemostat for 30 seconds simulating a soft tissue injury, and then 0.15 mL bone mixture suspended in PBS was injected using a 1-mL syringe and an 18-gauge needle in the area of the soft tissue injury of each thigh.

After 6 hours, thoracotomy (using anesthesia with pentobarbital and isoflurane) was performed and blood was collected via cardiac puncture. Blood samples were centrifuged at 5000 rpm for 10 minutes to separate the serum from cellular blood components and stored at -20°C until thawed for cytokine profile measurements. The left liver lobe and left lung were removed and

Fig. 1 A flow diagram illustrates the experimental design.

snap-frozen in liquid nitrogen for molecular assays. The systemic inflammation was evaluated in each mouse by measuring serum IL-6 and IL-10 levels, which were quantified with ELISA kits (R&D System Inc, Minneapolis, MN, USA). We similarly analyzed the role of OPN as a posttraumatic inflammation marker by quantifying it using OPN ELISA kits (R&D System Inc). Hepatocellular damage after trauma was assessed by measuring serum alanine aminotransferase (ALT) using a chemistry analyzer (Dri-Chem[®]; Fujifilm Corp, Asaka-shi, Saitama, Japan).

We quantified the MPO activity in lung tissue to assess remote organ damage and inflammation after the trauma applied in our experiment. Five milligrams of the snap-frozen tissue (from the left lung, lower lobe, anteromedial segment) was thawed and homogenized in a lysis buffer exactly as described by the manufacturer. MPO-enzyme-linked immunosorbent assay kits (Cell Sciences, Canton, MA, USA) were used to quantify the MPO activity in each lung. Two replicates of each assay were performed to have more reliable data.

Hepatic inflammation response is associated with nuclear factor (NF)- κ B activity [4]. We quantified the NF- κ B activity in liver tissue to assess remote organ damage and inflammation after the trauma applied in our experiment. Livers harvested at the conclusion of the experiment were thawed. Five milligrams of tissue (left lobe) was removed and the tissue of each mouse was homogenized in a buffer. We measured NF- κ B activity using electrophoretic mobility shift assays with nuclear extracts prepared from the tissue. Electrophoretic mobility shift assays were performed as described previously [21].

Data were expressed as the mean $\pm$ standard error of six to nine animals per group. Group comparisons were

assessed using ANOVA (Bonferroni) in normally distributed variables. Nonnormally distributed parameters were tested using the Kruskal-Wallis test. We used SigmaStat[®] Version 3.1 (SPSS Inc, Chicago, IL, USA) for statistical analyses.

Results

Both isolated bone injection and soft tissue injury showed minor effects on the systemic inflammatory response. The BF group (62 ± 24 pg/mL) and STI group (68 ± 52 pg/mL) demonstrated comparable mean IL-6 levels (Fig. 2). Moreover, serum IL-6 levels were comparable ($p = 0.711$) to the levels measured in the control group (14 ± 19 pg/mL). Similarly, isolated injuries caused no increase ($p = 1.0$) in IL-10 (BF: 19 ± 39 pg/mL; STI: 8 ± 4 pg/mL) (Fig. 3). Moreover, we found no increased OPN levels at 6 hours after traumatic stimulus ($p = 1.0$) (Fig. 4).

Fracture-associated soft tissue injury in combination with the exposure of bone components stimulated the posttraumatic inflammatory response but not the remote organ inflammation. The BF + STI group demonstrated the highest levels of IL-6 (189 ± 102 pg/mL, $p = 0.001$) and IL-10 (63 ± 45 pg/mL, $p = 0.009$). The STI group (66 ± 6 U/L) and BF + STI group (77 ± 14 U/L) showed an increase in ALT levels compared to both the control group (26 ± 2 U/L; $p < 0.001$) and the BF group (45 ± 12 U/L; $p = 0.002$) (Fig. 5). The STI and BF + STI groups showed an increased hepatic NF- κ B activation (Fig. 6). Whereas the BF + STI group demonstrated the highest band density among the groups, the control and BF groups demonstrated a

Fig. 2 A graph shows the IL-6 levels in the control, BF, STI, and BF + STI groups. Data are expressed as mean $\pm$ standard error of six to nine animals per group. The end point was 6 hours. The BF and STI groups demonstrated comparable IL-6 levels but were higher than the control group. The BF + STI group demonstrated the highest level of IL-6.

Fig. 3 A graph shows the IL-10 levels in the control, BF, STI, and BF + STI groups. Data are expressed as mean $\pm$ standard error of six to nine animals per group. The end point was 6 hours. The BF and STI groups showed only a slight increase in IL-10. The BF + STI group demonstrated the highest level of IL-10.

less pronounced hepatic inflammation response with low NF- κ B activity. Elevation of MPO activity was found in the BF + STI group (1.2 ± 0.4 ng/mL; $p = 0.044$) and the STI group (1.3 ± 0.7 ng/mL; $p = 0.039$) compared with the control group (0.5 ± 0.2 ng/mL) (Fig. 7).

Discussion

Severe long-bone fractures are frequently associated with adjacent soft tissue injuries. Both can result in severe

Fig. 4 A graph shows the OPN levels in the control, BF, STI, and BF + STI groups. Data are expressed as mean $\pm$ standard error of six to nine animals per group. The end point was 6 hours. The BF + STI group demonstrated a slight increase in OPN, whereas the other groups did not show a response.

Fig. 5 A graph shows the ALT levels in the control, BF, STI, and BF + STI groups. Data are expressed as mean $\pm$ standard error of six to nine animals per group. The end point was 6 hours. The STI and BF + STI groups showed a slight but significant increase in ALT levels compared to both the control and BF groups.

generalized inflammation and associated remote organ dysfunction. In our experiment, we mainly distinguished between soft tissue trauma and (hard or) bone tissue injury. The assessment of the systemic inflammatory status in patients with multiple injuries may be helpful for decision making. Using a mouse model, we therefore answered the following questions: (1) Does isolated fracture-associated bone liberation contribute to the systemic inflammatory response? And (2) does fracture-associated soft tissue injury in combination with the exposure of bone components affect the posttraumatic inflammatory response and remote organ inflammation?

Several limitations should be considered before interpretation of our results. First, in our bone injection and soft tissue injury model, the exact concentration of bone fragments and bone marrow injected into the injured thigh musculature was unknown. We standardized the number of

Fig. 6 NF- κ B activation in the control, BF, STI, and BF + STI groups is shown. The end point was 6 hours. The BF + STI group demonstrated the highest band density among the groups, the STI group showed an increased activation, and the control and BF groups demonstrated low activity. + = positive control.

Fig. 7 A graph shows the MPO activity in the control, BF, STI, and BF + STI groups. Data are expressed as mean $\pm$ standard error of six to nine animals per group. The end point was 6 hours. The BF + STI and STI groups showed elevated MPO activity compared with the control group.

long bones (femur and tibia were used) and volume of PBS to minimize this effect. These injections did reproduce the inflammatory response in this model. In previous studies of this model [7, 17, 22, 27], we tested and standardized the experimental protocol and showed the reproducibility of the systemic response after bilateral femur fractures [22]. Second, vascular and nerve injuries might be present in our soft tissue injury model and affect the results of our experiment. To minimize these effects, a standardized soft tissue injury was used in each animal. Moreover, at the end of the experiment, we performed a necropsy to rule out the presence of severe hematoma or incorrect tissue injury. Third, in this experiment, only one time point (6 hours) was assessed. This time point was chosen due to the peak of proinflammatory cytokine response and increased inflammation within the lung and liver after trauma in mice [5].

However, no conclusions can be drawn regarding the dynamics of the posttraumatic inflammatory response for the experimental groups in this study. Finally, an immune response from allogeneic tissue transplantation may take place in our experiment. The C57/BL6 mice strain was used in our study. This is an inbred mouse stem (> 50 generations) and mice are nearly identical to each other in genotype. Moreover, immune response in allogeneic tissue transplantation is a chronic inflammation process. In our experiment, we mainly focused on acute inflammation patterns.

Fracture results in deterioration of the bony architecture, rupture of the periosteal tissue, and vascular injury, leading to the development of hematoma. The hematoma is rich in activated cells of bone marrow, immune cells, tissue debris, and preformed cytokines [14, 26, 32]. Multiple long-bone fractures reportedly associated with severe changes in the innate and adaptive immune system [9]. Pro- and anti-inflammatory cytokines (IL-6, IL-8, IL-1, TNF- α , IL-10, etc) are systemically released and are associated with the development of remote organ injury and immunodysfunction [15]. Measurements of the systemic inflammatory response can be useful adjuncts to clinical evaluation in severely injured patients [2, 25]. Moreover, treatment strategies (damage control surgery versus early total care) are known to also affect inflammation parameters such IL-6 or IL-10 [11, 25].

Our findings suggest both isolated bone injection and soft tissue injury are necessary to stimulate the systemic inflammatory response. Other investigators have also demonstrated minor injuries of the lower leg do not demonstrate any relevant systemic immunologic changes [33]. While a systemic release of muscle-specific proteins was detected, systemic cytokine expression and the response of immune-competent cells (splenocytes and macrophages) were not elevated [33]. Minor soft tissue injury appears to be associated with the systemic liberation of muscle debris without the activation of immune cells. Whether inflammation thresholds, injury differences in adjacent structures (eg, vasculature or nerves), or alterations in perfusion between minor and severe soft tissue injury exist has yet to be analyzed. In addition, large amounts of comminuted bone fragments with immunologic properties were injected in our experiment. The mechanisms by which bone components stimulate acute systemic inflammation after long-bone fractures are not well understood. However, it appears that bone fragment may affect the severity of systemic inflammation.

We found substantial elevation of both inflammatory markers (IL-6 and IL-10) after soft tissue injury in combination with bone component injection. These results indicate the combination may induce a cumulative pro- and anti-inflammatory burden. However, the dynamics of the inflammatory process within the first 6 hours should be studied to confirm this presumption. Analyzing remote organ inflammation in each group did not identify the

distinct patterns we observed in the systemic cytokine response. The injection of bone solution in combination with soft tissue injury was associated with serum ALT levels and NF- κ B expression comparable to those in mice with isolated soft tissue injury. According to our results, further fracture-associated factors are necessary to induce major liver or lung injury. Bleeding in combination with a fracture reportedly does not change the systemic cytokine pattern of IL-6 and IL-10 [27]. However, hypovolemia apparently increases the degree of lung injury [27]. This may be a result of the vulnerability of the lung tissue due to high blood circulation and accumulation of systemically released inflammatory mediators and immune cells. Furthermore, Schemitsch et al. [31] reported lung injury without associated systemic inflammation might be related to the liberation of fat content after long-bone fractures.

OPN, a component of extracellular bone matrix, is a protein with diverse functions in the immune system. We observed a slight trend toward elevated serum OPN in mice subjected to both injuries, but whether the trend can be confirmed and its importance are unclear. The reason for the low levels may be due to the short observation period (end point 6 hours) in our study. Mori et al. [23] reported OPN peaks after 1 to 3 days after injury when analyzing wound granulation tissue. Therefore, further long-term investigations may give us more insight about the inflammatory patterns of OPN. In addition, since OPN is usually expressed by fibroblasts, activated macrophages, and lymphocytes at the injury site, local measurements after femur fractures could be beneficial as well [34].

Our observations suggest both bone components and soft tissue injury contribute to the systemic inflammatory response after fracture. However, this pattern was not observed regarding remote organ injury. According to our results, further fracture-associated stimuli appear to affect the severity of the organ damage after long-bone fractures. This phenomenon can be due to bleeding and possible additional expression or release of various mediators (eg, intramedullary fat) that are absent in our models. Further investigations are necessary to understand the role of bone fragments in induction of the systemic inflammatory response and generation of remote organ damage.

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