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Models Of Lower Extremity Damage In Mice: Time Course of Organ Damage & Immune Response

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Abstract

Background—Posttraumatic inflammatory changes have been identified as major causes of altered organ function and failure. Both hemorrhage and soft tissue damage induce these inflammatory changes. Exposure to heterologous bone in animal models has recently been shown to mimic this inflammatory response in a stable and reproducible fashion. This follow-up study tests the hypothesis that inflammatory responses are comparable between a novel trauma model (“pseudofracture”, PFx) and a bilateral femur fracture (BFF) model.

Materials and Methods—In C57BL/6 mice, markers for remote organ dysfunction and inflammatory responses were compared in 4 groups (control/sham/BFF/PFx) at the time points 2, 4, and 6 hours.

Results—Hepatocellular damage in BFF and PFx was highly comparable in extent and evolution, as shown by similar levels of NFκB activation and plasma ALT. Pulmonary inflammatory responses were also comparably elevated in both trauma models as early as 2h after trauma as measured by myeloperoxidase activity (MPO). Muscle damage was provoked in both BFF and PFx mice over the time course, although BFF induced significantly higher AST and CK levels. IL-6 levels were also similar with early and sustained increases over time in both trauma models.

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Conclusions—Both BFF and PFx create similar reproducible inflammatory and remote organ responses. PFx will be a useful model to study longer term inflammatory effects that cannot be studied using BFF.

Keywords

long bone fracture; soft tissue injury; pseudofracture; liver dysfunction; acute lung injury; systemic inflammation

Introduction

Trauma accounts for a major percentage of deaths (1) with late deaths accounting for about 14% to 20% of all trauma-related deaths (2–4). Immune responses triggered by trauma play a pivotal role in the development of subsequent remote organ damage (5–8). Both inflammatory and anti-inflammatory pathways are activated - concepts which are clinically known as severe inflammatory response syndrome (SIRS) and counterregulatory anti-inflammatory response (CARS). Models employed to study the immune response after trauma have used hemorrhagic shock, soft tissue injury and fractures (9–14). Fractures are a common feature of severely injured patients and known to induce posttraumatic systemic inflammation and organ damage such as acute lung injury or liver dysfunction (10,14–17).

Experimental models in rodents that incorporate long-bone fractures are limited because they cannot be easily extended beyond a few hours due to ethical and IACUC restrictions. In order to study the delayed immunologic consequences of peripheral skeletal trauma, we recently developed a pseudofracture model (PFx) in mice (18). This model was successfully used to show the pivotal role of TLR4 in signaling pathways of tissue damage leading to systemic inflammation and acute lung injury. However, further evidence was still needed to establish whether the PFx model replicates the initial inflammatory response induced by long bone fractures in order for the model to be used in further longer term experimental studies.

Our hypothesis was that early innate immune responses after pseudofracture would follow a similar pattern to those responses elicited by bilateral femur fracture. In detail, we hypothesized that the magnitude and the time course of organ damage and inflammatory response is comparable in the PFx model versus a model of bilateral femur fracture (BFF). We assessed parameters for muscle, lung and liver damage, as well as markers of systemic inflammation. In order to evaluate the development of immune responses over time in both models, we collected data at time points of 2, 4 and 6h. Our results show that the immune response in both models develops similarly, which validates further longer-term studies using the pseudofracture model. These later responses could not be tested using the current BFF model because of IACUC restrictions on experiment length.

The mechanisms leading to innate immune responses following trauma and long bone fracture are wide-ranging and multifactorial. In this study we have determined that injection of crushed bone into the thigh muscle of mice is one mechanism that can produce similar early innate immune responses to bilateral femur fracture. This is an important finding that will lead to advances in the study of femur fracture over longer time points.

Materials and Methods

Animal Care

All experimental protocols were approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh, and experiments were carried out in accordance

with the regulations regarding the care and use of experimental animals published by the National Institute of Health. Male C57BL/6 mice (Charles River Laboratories International, Wilmington, MA), 7 to 11 weeks of age and weighing 20 to 30g, were used. All animals were acclimated for at least 7 days in our animal facility with free access to food and water. Anesthesia during experiments was performed through inhalation of Isoflurane (Abbott Laboratories, Chicago, IL) and injection of Pentobarbital Sodium (70 mg/kg, i.p., Ovation Pharmaceuticals, Deerfield, IL); repeated doses of Pentobarbital Sodium (10 mg/kg, i.p.) were given as necessary throughout the course of the experiment.

Experimental Groups

Mice (n=71) were divided into 4 groups. In order to obtain physiological baseline levels, control group mice (n=5) were immediately sacrificed under inhalation anesthesia with no procedures performed. At each of the time points 2, 4, and 6h, 22 mice were euthanized; the mice were distributed among the groups as follows: sham (S, n=6), bilateral femur fracture (BFF, n=8), and pseudofracture (PFx, n=8). Sham mice were anesthetized and immobilized for the course of the experiment in the same way as the other treatment groups. In both the BFF and the PFX groups, soft tissue injury was induced by clamping both thigh muscles with a large hemostat for 30sec. BFF mice underwent additional induction of bilateral femur fractures, performed manually with the aid of hemostats. PFX mice instead received an i.m. injection of a crushed-bone solution into each thigh using a 20G needle. The volume chosen is the maximum volume (0.15mL) allowed by IACUC protocols for intramuscular injections in mice.

Preparation of the Crushed-Bone Solution

The crushed-bone solution for injection was prepared as described previously (18). A mouse was sacrificed, and both femurs and tibias were extracted under sterile conditions at room temperature and put in a tube on ice. Within minutes of removal of the bones, the following steps were performed on ice: The bones were crushed by mortar and pestle under a sterile hood; saline (total 2 mL for 4 bones) was added and the mixture was homogenized for 2min until an injectable solution was obtained that was drawn up into syringes and placed at 4°C. Each experimental day the solution was freshly prepared and kept cold for a maximum of 1h until further use. Each homogenate was monitored for sterility by culturing one drop of the solutions on sheep blood tryptic soy agar blood plates at 37°C; none of the batches showed contamination. Specifically, these plates were used to detect the largest possible range of bacteria (fastidious or slow-growing aerobic and anaerobic bacteria, as well as hemolytic bacteria) that might be cultured from the bone homogenates.

Blood and Tissue Collection

Mice were anesthetized through inhalation of isoflurane and euthanized by opening the chest and collecting whole blood by cardiac puncture. Immediately after cardiac puncture the liver and the lungs were collected, snap frozen in liquid nitrogen and stored at -80°C until further analysis. The collected heparinized blood samples were centrifuged at 2300xg for 10 min. Plasma samples then were aliquoted, with one aliquot immediately used for quantification of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine phosphokinase (CK) levels using the Dry-Chem Veterinary Chemistry Analyzer (HESKA, Loveland, CO; slides from Fujifilm Corporation, Asaka-shi Saitama, Japan). The other plasma aliquots were stored at -80°C until further analysis of cytokine levels with the Luminex multiplexing platform (MiraiBio, Alameda, CA) using mouse cytokine bead sets for Interleukin(IL)-6 (Invitrogen, San Diego, CA). IL-6 levels were validated by ELISA to ensure comparability of the data, and no significant differences were found between ELISA results and Luminex results.

Skeletal Muscle Histology

Immediately after euthanasia, thigh muscles were harvested from the mice in the control group and the 6h BFF and 6h PFx groups for haematoxylin and eosin (H&E) staining. Briefly, tissues were fixed in 2% paraformaldehyde for 4h and then transferred through a series of alcohol processing steps (70 to 100%), followed by tissue incubation in xylene. Samples were embedded in paraffin, sectioned at 5 μ m, and stained with H&E. Images were taken on a Olympus Provis light microscope (Malvern, NY) with 100x magnification.

Preparation of Nuclear Extracts from Liver Tissue and Electrophoretic Mobility Shift Assay (EMSA)

NF κ B DNA-binding activity was determined by EMSA on nuclear extracts which were obtained from portions of 50mg snap frozen liver tissue. 500 μ L of Buffer A (10mM HEPES at pH 7.9, 1.5mM MgCl₂, 10mM KCl, 0.5% NP-40, 0.5mM dithiothreitol [DTT], 0.1mM PMSF; Leupeptin, Aprotinin, Pepstatin A and Chymostatin each at 2 μ g/mL) was used for the homogenization process in a glass dounce homogenizer, and samples were incubated on ice for 15min and then centrifuged at 2300xg for 5min; subsequently the supernatant was removed, the pellet resuspended in Buffer A and kept on ice for 10min. Nuclear proteins were extracted by gentle resuspension of the nuclei in 40 μ L of 3 parts buffer C (20mM HEPES at pH 7.9, 25% glycerol, 1.5mM MgCl₂, 45mM KCl, 0.2mM EDTA, 0.5mM DTT, 0.1mM PMSF; Leupeptin, Aprotinin, Pepstatin A and Chymostatin each at 2 μ g/ml) together with 1 part buffer D (as for buffer C except 400mM KCl) added in a dropwise fashion. Samples were kept on ice for a further hour, followed by centrifugation at 13000xg for 15min and collection of the supernatants. Protein concentration was determined by BCA protein assay according to manufacturer's instructions (Pierce, Rockford, IL). Double-stranded NF κ B-specific oligonucleotide was end-labeled by incubation with [γ -³²P]ATP using T4 polynucleotide kinase (U.S. Biochemicals, Cleveland, OH) at 37°C for 30min, and purified on a G-50 Sephadex column at 3000xg for 10min. Nuclear proteins (5 μ g per well) were incubated with 50,000cpm of ³²P-labeled oligonucleotide for 45min at room temperature in a reaction mixture containing 1 μ g poly(dI-dC), 12.5mM Tris-HCl (pH 7.5), 50% glycerol, 0.25mM EDTA, 1.25% NP40, and 0.25mM DTT (final volume 20 μ L). The DNA-protein complexes were resolved on a 4% nondenaturing polyacrylamide gel in 0.5 x Tris-borate-EDTA (TBE) buffer. The gels were dried using a vacuum-assisted gel-dryer and then subjected to autoradiography using the Cyclone Storage Phosphor Screen System (Packard Instrument Co, Meriden, CT).

Lung Myeloperoxidase Activity

As a marker for neutrophil (PMN) cell infiltration myeloperoxidase activity (MPO) activity in lung tissue lysates was determined with a mouse MPO ELISA kit (Cell Sciences, Canton, MA) according to the manufacturer's protocol. Briefly, portions of the frozen lungs (about 10mg) were homogenized in lysis buffer with a Tissue Tearor machine (Biospec Products Inc., Bartlesville, OK) and then centrifuged at 1500xg for 15min. The supernatant was diluted 5-fold, and its MPO activity determined and then standardized to the respective protein concentration (BCA Protein Assay Kit, Pierce, Rockford, IL).

Splenocyte Proliferation Assay

Spleens were harvested from control (unmanipulated) mice and mice at 48h after pseudofracture. Single cell splenocyte suspensions are prepared by digestion of spleens with collagenase D (Roche) followed by mechanical tissue break-up through a 70 μ m mesh strainer. Splenocytes were then washed with PBS and centrifuged at 1200 rpm for 10min. Erythrocytes were lysed with RBC lysing buffer (Sigma) and cells washed again in PBS. The cell pellet was resuspended in RPMI 1640 (containing 10% FBS, L-glutamine, pen/

strep, essential AA, sodium pyruvate, 2ME) to a final concentration of 1×10^6 cells/ml. Cell viability of 90% was confirmed by trypan blue staining. Splenocytes were cultured for 72h at 37°C and stimulated with concanavalin A (2.5µg/mL – GE healthcare) with tritiated thymidine (1µCi/well - Perkin Elmer) added for the last 18h of culture. Proliferation (thymidine incorporation) was measured at the end of culture.

Statistical Analysis

Statistical Analysis (STATA 10, StataCorp LP, College Station, TX) was performed by a professional statistician of the Clinical Research Office for Surgery and Trauma (CROST) of the Department of Surgery, University of Pittsburgh. The data was subjected to appropriate transformations in order to achieve normal distribution and meet assumptions of ANOVA; post-hoc-tests according to Sidak were performed. Nonparametric tests (Wilcoxon rank sum test) were utilized when complete assumptions for an ANOVA were not met. Results are presented as mean $\pm$ standard error (SEM) with differences being accepted as statistically significant if the p-value was less than 0.05.

Results

BFF and PFx induce similar inflammatory responses remote from site of injury

Peripheral tissue trauma induces responses remote from the site of injury in the form of inflammation and damage in central organs such as the liver and lungs. We measured ALT, NFκB DNA-binding activity, and pulmonary MPO in order to determine whether BFF and PFx induced similar changes to each other in the liver and lungs over time and also to similar magnitudes. Plasma, liver and lung were isolated at 2, 4, and 6h following injury. As shown in figure 1A, increases in plasma ALT occurred in both models to a similar level above sham treated animals at all three time points. The modest increases reflect the minor level of liver damage known to occur in femur fracture models (12, 14). Nuclear NFκB DNA-binding activity was also assessed in the liver (Fig. 1B). Again, both BFF and PFx induced similar increases in NFκB activation at the three time points measured. Lung responses were assessed by measuring PMN accumulation via MPO activity (Fig. 2). BFF and PFx induced near identical increases in lung MPO activity by 2h. These increases persisted to the 6h time point. Sham treatment did not increase lung MPO activity over the baseline levels. These data suggest that responses remote from the site of injury are similar in the BFF and PFx models. Liver and lung histology showed no obvious signs of organ damage at these early time points and this is consistent with findings in previous investigations using the more severe hemorrhagic shock model (19).

BFF and PFx are associated with similar increases in AST and CK

When skeletal muscle is damaged the intracellular enzymes, AST and CK, leak into the circulation. To estimate the magnitude of muscle damage in the two models, we measured circulating AST and CK levels. As shown in figure 3, both models induced significant increases over controls and sham animals in both enzymes. AST levels remained elevated to a similar level at all three time points, while CK peaked at 2h and then gradually declined. Interestingly, anesthesia and immobilization (sham group) causes a gradual increase in both AST and CK levels over time. This indicates that immobilization alone can increase skeletal muscle enzyme leakage. Also of note, BFF induced observable increases in AST and CK levels when compared to PFx, although these differences were statistically significant only at an early time point (2h) for AST levels. This could reflect the consequences of the loss of stability provided by the initial fracture with greater ongoing trauma to the muscle, or the increased trauma to soft tissue endured when producing BFF with hemostats.

BFF and PFx induce similar levels of skeletal muscle damage

Comparison of histological slides of skeletal muscle from the thighs (Fig. 4) shows evidence of muscle trauma after both BFF and PFx. Histological changes are very similar between experimental groups with no overall structural changes to the skeletal muscle organization at 6h after either BFF or PFx. However, after crush damage in both models (Fig. 4B, 4D) a widening of the capillaries with extravasation of erythrocytes is seen. Sections through the hematoma show large numbers of erythrocytes (Fig. 4D, 4E, 4F) with the PFx group showing evidence of tissue edema consistent with a volume of injected material in the local tissue area (Fig. 4D).

BFF and PFx induce similar increases in circulating IL-6 levels

Circulating plasma IL-6 levels have been shown to correlate with the magnitude of the tissue trauma (12). We found that both BFF and PFx induced similar increases in plasma IL-6 by 2h, and IL-6 remained elevated to 6h (Fig. 5). Sham treatment did not induce increases in IL-6 over baseline. Therefore, the systemic response as measured by plasma IL-6 levels is similar between the two models for the first 6h.

PFx induces immunosuppression at 48h

Previous studies have shown immunosuppression following severe trauma and after femur fracture (20). This occurs at 24–48h after injury. In order to confirm similarities in immunosuppression between PFx and BFF we determined splenocyte proliferation at 48h after PFx compared with control (unmanipulated) mice. As expected we showed a significant decrease in splenocyte proliferation in PFx mice compared with controls (Fig.6) as well as decreased production of IFN γ . These data suggest similar immunosuppression following PFx as previously reported with BFF.

Discussion

Posttraumatic systemic inflammation and subsequent remote organ damage, as well as delayed immune dysfunction, are clinically highly relevant and associated with high morbidity and mortality (21,22). Underlying mechanisms have been studied in a variety of experimental animal models, including femur fracture (11,12,17,23–25). However, fracture models in mice face limitations especially when planning long term studies. To facilitate studies addressing long term immune responses, we recently developed a PFx model in mice, simulating bone fracture by exposing injured muscle to crushed-bone components (18). This novel model has been successfully applied to elucidate the role of TLR4 in posttraumatic inflammation and lung damage (18). Here we carried out studies to directly compare the initial inflammatory responses of BFF and PFx. More precisely, we hypothesized that the magnitude and the time course of organ damage and inflammatory response is comparable in these both models.

We show here that there are strong similarities between BFF and PFx in terms of time course of inflammatory changes and extent of remote organ dysfunction and damage at early time points, as well as preliminary data to show late immunosuppression after PFx similarly to that seen by others after BFF (20). Liver damage was increased similarly and occurred remarkably early in both models. The early increase in liver damage is mirrored by increased NF κ B activation. NF κ B is known to promote the transcription of proinflammatory mediators, such as the cytokine IL-6, after it is translocated to the nucleus. We have previously shown that hepatic NF κ B activation is induced after BFF (9,10), and our data from this study in BFF mice are consistent with those findings. In the present study, PFx shows similar levels of NF κ B activation and liver damage, suggesting similar liver inflammatory signaling is activated.

In clinical settings of posttraumatic remote organ damage another early target organ for inflammation in addition to the liver is the lung, with acute respiratory distress syndrome (ARDS) often developing in trauma patients (5,7,21). In experimental settings, pulmonary MPO activity is measured frequently as a parameter for PMN infiltration and thus pulmonary inflammation (18,26,27). Excessive activation of PMNs can also lead to destruction of tissue. A study performed by Kobbe *et al* (14) demonstrated an increase in pulmonary MPO activity by 1h after induction of BFF in mice. Concordant with these findings, we found pulmonary MPO activity to be increased in our earliest measured time point of 2h after trauma, and again, this was similar for both experimental groups, BFF and PFx.

There were also strong similarities between BFF and PFx in terms of time course of inflammatory activation and local damage to the muscle. In both groups, similar increases in AST and CK levels showed the akin magnitude of the sustained injury. However, BFF demonstrated a tendency towards initially higher levels for AST and CK with significantly higher AST levels at 2h only. The observable differences are not unexpected given the increased force required to break the femurs and the likely extra tissue damage that occurs in the BFF group. We had expected that the increased soft tissue injury of BFF would lead to more exaggerated differences in inflammatory response between the models as it has been shown that soft tissue injury in addition to fractures results in a stronger inflammatory response and more severe hepatic inflammation/dysfunction than soft tissue injury or fracture alone (12). Interestingly, PFx led to equivalent inflammatory responses including circulating IL-6 levels, which are an established marker for the systemic inflammatory response overall. Equivalent immune responses therefore make PFx a good model to use in situations where BFF cannot be used. However, possible modifications of the PFx model in future studies with more extensive muscle injury may be of interest in order to make the models even more similar.

Circulating IL-6 is an established marker for systemic inflammatory responses. Elevated levels of IL-6 after trauma mirror the magnitude of the insult (28), and the clinical course and outcome of trauma patients may be predicted by measuring posttraumatic IL-6 levels (29). As a cytokine, IL-6 serves as pro-inflammatory mediator, leads to B and T cell differentiation, and induces hepatic acute phase protein release (21). Previously it has been demonstrated in several animal models that soft tissue injury and/or fractures increase circulating IL-6 levels (9,12,14). Our findings in BFF and PFx injured mice are consistent with these studies, where observed IL-6 levels are elevated early and remain elevated over the full time course of 6h, indicative of ongoing inflammation in both models.

When interpreting the results of this study there are limitations that need to be addressed. In order to further characterize the time course changes within individual mice in both models, repetitive blood withdrawals would need to be taken from each mouse at each time point. However, the low blood volume of mice does not allow repetitive blood withdrawals without hemodynamic effects. In order to avoid these and other confounding factors, we randomized mice to the experimental groups and thus harvested separate mice at each time point. Nevertheless, the fact that no statistically significant difference could be found between sham and control groups, or in between sham groups across all parameters and time points, provides internal validation for the procedures applied in our models.

Another methodological aspect of this study that should be taken into consideration involves the immunogenic effects of injection of crushed-bone into muscle tissue. In transplant surgery, skeletal allografts may be submitted meticulously to a variety of procedures (such as freezing, demineralisation, boiling, autoclaving) to reduce immunogenicity for the host (30). In our PFx model we injected full bone suspension specifically with the aim of

inducing reproducible levels of inflammation within the PFX group. Such reproducible inflammatory responses are achieved by using “syngeneic grafts” from mice within the same inbred strain as the recipient mice. We have previously shown that the inclusion of both bone and cellular elements gives the greatest inflammatory response when injected into muscle (18). We have also previously shown that TLR4 is instrumental in the production of immune responses produced in both BFF (11) and PFX (18). Taken together, data from this present study as well as our previous studies strongly suggest that the molecular stimulation of the immune response is similar between BFF and PFX and this supports the potential use of PFX in longer time point studies where BFF cannot be used.

In summary, both the BFF and the PFX models result in peripheral injury-mediated systemic inflammatory responses and early increases in markers of organ damage at remote sites. Time course and extent of hepatocellular damage, muscle injury, and lung damage are similar in mice undergoing either BFF or given injection of crushed-bone components into injured muscle. This study therefore confirms the potential usefulness of the PFX model to study later time points after trauma. This model may allow us to study the longer term effects of trauma on inflammatory responses and will therefore likely be important in providing us with improved understanding of the mechanisms of immune deficiencies seen in trauma patients.

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Figure 1.
Hepatocellular damage in mice at 2, 4, and 6h time points in control (n=5), sham (S, n=6), bilateral femur fracture (BFF, n=8) and pseudofracture (PFx, n=8) groups. **A:** Plasma ALT levels. Results show mean $\pm$ SEM; *= p <0.05 vs control; #= p <0.05 vs sham). **B:** EMSA showing NFκB activation in liver tissue (representative result of three assays).

Figure 2.

Pulmonary myeloperoxidase (MPO) activity at 2, 4, and 6h time points in control (n=5), sham (S, n=6), bilateral femur fracture (BFF, n=8) and pseudofracture (PFx, n=8) groups. MPO activity was standardized to protein concentration of lung tissue lysates. Results show mean $\pm$ SEM; * = $p < 0.05$ vs control, # = $p < 0.05$ vs sham.

Figure 3.
Muscular damage in mice at 2, 4, and 6h time points as determined by plasma levels of **A:** CK and **B:** AST in control (n=5), sham (S, n=6), bilateral femur fracture (BFF, n=8) and pseudofracture (PFx, n=8) groups. Results show mean $\pm$ SEM; + = p<0.05 vs PFx, * = p<0.05 vs control, # = p<0.05 vs sham.

Figure 4. Histological changes in skeletal muscle 6h after BFF and PFx
A: Control; **B:** PFx; **C:** BFF; **D:** PFx through hematoma; **E and F:** BFF through hematoma.

Figure 5.

Inflammatory response as measured by IL-6 levels at 2, 4, and 6h time points in control (n=5), sham (S, n=6), BFF and PFX mice (n=8 per group). Levels for Control and Sham at 2h and 6h time points are not detectable (ND; level of detection: 5.2 pg/mL). Results show mean $\pm$ SEM; * = $p < 0.05$ vs control, # = $p < 0.05$ vs sham.

Figure 6. Immunosuppression after 48h of PFx

Splenocyte proliferation in response to Concanavalin A treatment in control (n=8) and PFx (n=4) mice at 48h after injury. Results show mean $\pm$ SEM; * = $p < 0.05$ vs control.