Published in final edited form as:

J Trauma. 2011 April ; 70(4): 948–953. doi:10.1097/TA.0b013e3181e1d27b.

The Effects of Injury Magnitude on the Kinetics of the Acute Phase Response

Graciela Bauzá, MD

Departments of Surgery, Boston University School of Medicine, Boston, Massachusetts

Glenn Miller, MD

Departments of Surgery, Boston University School of Medicine, Boston, Massachusetts

Neema Kaseje, MD

Departments of Surgery, Boston University School of Medicine, Boston, Massachusetts

Nathan A. Wigner, PhD

Department of Orthopaedics, Boston University School of Medicine, Boston, Massachusetts

Zhongyan Wang, MD, PhD

Departments of Surgery, Boston University School of Medicine, Boston, Massachusetts

Louis C. Gerstenfeld, PhD

Department of Orthopaedics, Boston University School of Medicine, Boston, Massachusetts

Peter A. Burke, MD

Departments of Surgery, Boston University School of Medicine, Boston, Massachusetts

Abstract

Background—The acute-phase response (APR) is critical to the body's ability to successfully respond to injury. A murine model of closed unilateral femur fractures and bilateral femur fracture were used to study the effect of injury magnitude on this response.

Methods—Standardized unilateral femur fracture and bilateral femur fracture in mice were performed. The femur fracture sites, livers, and serum were harvested over time after injury. Changes in mRNA expression of cytokines, hepatic acute-phase proteins, and serum cytokines overtime were measured.

Results—There was a rapid and short-lived hepatic APR to fracture injuries. The overall pattern in both models was similar. Both acute-phase proteins' mRNA (fibrinogen- γ and serum amyloid A-3) showed increased mRNA expression over baseline within the first 48 hours and their levels positively correlated with the extent of injury. However, increased severity of injury resulted in a delayed induction of the APR. A similar effect on the gene expression of cytokines (interleukin [IL]-1 β , IL-6, and tumor necrosis factor- α) at the fracture site was seen. Serum IL-6 levels increased with increased injury and showed no delay between injury models.

Conclusions—Greater severity of injury resulted in a delayed induction of the liver's APR and a diminished expression of cytokines at the fracture site. Serum IL-6 levels were calibrated to the extent of the injury, and changes may represent mechanisms by which the local organ responses to injury are regulated by the injury magnitude.

Keywords

Acute phase response; Injury severity; Femur fractures; Gene expression; Cytokines

The injury response is a complex physiologic process and is characterized by the acute-phase response (APR). Although the APR has evolved as a survival mechanism, maintenance of this inflammatory state over long periods of time may have negative clinical consequences.^{1,2} The liver is a primary mediator of the APR and alters its biosynthetic profile to produce acute-phase proteins (APPs) with the induction of “positive APPs” (e.g., fibrinogen- γ , serum amyloid A [SAA]-3, and C-reactive protein) and reduction of “negative APPs” (e.g., albumin and prealbumin) as a response to injury.³ Moreover, the injury response is characterized clinically by two distinct phases: the ebb and flow phases. This phenomenon first described by Cuthbertson⁴ in the 1930s describes a process in which an organism initially responds to injury by an ebb phase characterized by hypometabolism; poor tissue perfusion; mild protein catabolism; and low energy expenditure. After successful resuscitation, the organism enters the flow phase where it is hypermetabolic; tissue perfusion is normalized; and protein synthesis and catabolism is increased as is energy expenditure. Descriptions of these phases were originally based on observation of orthopedic trauma patients, although it would seem reasonable to extrapolate the same theoretical construct to all types of injury or critical illness, as is the case with the APR.^{5–7} Despite decades of research, the regulatory mechanisms of the ebb and flow phases remain incompletely elucidated. To date, it has been suggested that the intensity of injury suffered by the organism may play a role in the regulation of the ebb and flow phases of the injury response.⁵

In addition to the liver's response after injury, it is postulated that the wound releases mediators that act both locally and systemically as part of the injury response.⁸ Specifically, it has been observed that the release of interleukin (IL)-6 is an important part of the systemic response to injury. This cytokine has a myriad of effects after injury and is notable as being a potent inducer of the hepatic APR.^{9–12} Unlike other injury models, the simple closed femur fracture model is not confounded by the introduction of significant bacterial contamination.¹³ This model system also provides a consistent and quantifiable level of injury. Thus, the extent of injury can be varied by comparing less intense unilateral femur fractures (UFFs) to more extensive amounts of injury by generating bilateral femur fractures (BFFs). By using a murine femur fracture model, we examined the kinetics of the liver's injury response in comparison with wound proinflammatory cytokine gene expression produced at the site of injury after different intensities of injury. The level of systemic response was examined by the assessment of IL-6 protein levels in the serum. Our goal in these experiments was to assess differences at the molecular level in the kinetics of the hepatic APP mRNA response and determine how local expression of cytokine mRNA production was affected by the extent of injury as modeled by UFF and BFF. We hypothesized that the bilateral injury would result in a greater APR and more production of proinflammatory cytokines than the UFF. Furthermore, we hypothesized that these changes in injury response with differences in injury intensity are indicative of an underlying mechanism by which the ebb and flow phases of the injury response are regulated.

MATERIALS AND METHODS

Animals and Femur Fracture Models

Twenty-gram C57B1/6 male mice were housed in the animal facility. The experimental design and methods were approved by the Institutional Animal Care and Use Committee. At least 48 hours was allowed for acclimatization, and the mice had free and unlimited access

to food and water. On the day of the experiment, after anesthesia, intramedullary pins were placed in a retrograde fashion in unilateral or bilateral femurs of the mice. Animals were then placed on a “fracture apparatus” and standardized closed femur fractures were generated as described.¹³ Roentgenograms were performed to confirm both fracture and fixation (Fig. 1). Animals were subsequently killed at time points 6, 12, 24, and 48 hours after injury; livers, serum, and femurs were immediately harvested and stored at -80°C .

In the two fracture models (unilateral and bilateral), the mice were not given any resuscitation. There was no mortality in either injury models, and the animals were observed to resume ad libitum food consumption and ambulate shortly after recovery from anesthesia in both groups. At the time of harvest, tissues appeared well perfused, and there were no significant differences seen between the two injury groups.

Total RNA Isolation and mRNA Expression Analyses

Total RNA was extracted with Trizol (Life Technologies, Rockville, MD) according to the manufacturer's instructions. Gene expression of APR genes, fibrinogen- γ and SAA-3 in the liver tissue and IL-1 β , IL-6, and tumor necrosis factor (TNF)- α in the fracture hematoma were quantified by real-time polymerase chain reaction assays as described by Wang and Burke¹⁴ and Bais et al.¹⁵ using commercially purchased Taqman primer sets (Applied Biosystems, Foster City, CA). RNA samples were analyzed from at least three separate animals. The real-time polymerase chain reaction assays were run in triplicate.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay was performed after the manufacturer's instructions (eBioscience, San Diego, CA) to measure serum levels of IL-6.

Statistical Analyses

Statistical analyses were performed using analysis of variance (SAS, Cary, NC). Statistical significance was set at $p = 0.05$.

RESULTS

mRNA Expression of APR Genes in Liver Tissue in Response to Femur Fracture

Magnitude of APR induction by injury and its relationship to injury severity was tested using the UFF and BFF models because it represents a dichotomous break point in injury severity. To examine the effect of injury magnitude on APR gene expression, the levels of two classic markers of the APR (fibrinogen- γ and SAA) were examined in liver tissue. In UFF animals, mRNA expression of fibrinogen- γ showed significant increases in its expression ($p < 0.0003$) levels at 6 hours (~3 fold that of controls) with peak levels seen at 12 hours (~4 fold of controls) postinjury. Although the levels remained slightly increased over the next 48 hours, these were not significantly different from the control. In BFF animals, a similar pattern as UFF was observed; however, the induction of the APR was significantly delayed when compared with the UFF and the peak of activity was significantly greater (~25%) than the UFF ($p < 0.02$; Fig. 2, A).

Almost identical results were observed from SAA. In the animals with UFF, SAA mRNA expression rose rapidly reaching its peak at 6 hours and was six times greater than control levels ($p < 0.04$). Levels slightly decreased until 24 hours but remained significantly increased over the control levels with a return to the baseline 48 hours (Fig. 2, B). In contrast, BFF animals showed a small increased in there expressed levels at 6 hours, reached their peak levels at 12 hours, and remained slightly higher than the unilateral levels at 24 hours. All these levels were significantly greater than the controls ($p < 0.04$; Fig. 2, B). SAA

results showed similar kinetics and magnitude patterns to that of fibrinogen- γ mRNA and again illuminate that increase in injury intensity results in a delay in onset, a greater magnitude, and a more prolonged APR in the liver.

The mRNA Expression of Cytokines at the Fracture Site

To determine the molecular kinetics of cytokine production to injury magnitude at the injury site and identify potential systemic feedback on the local production of these cytokines in response to the extent of trauma, three major inflammatory cytokines (IL-1 β , TNF- α , and IL-6) that are produced in response to injury were examined. As shown in Figure 3, all three cytokines showed comparable responses to the extent of injury, with the unilateral injured groups showing significantly greater cytokine expression than the bilateral injury groups. Interestingly, each of these cytokines showed differing kinetics and folds of induction in their responses. Both IL-1 β and TNF- α reached maximal levels of increased expression within 6 hours after injury and remained increased through 12 hours in the unilateral groups, after which the levels fell back to near baseline. In contrast, the TNF- α level in bilateral group reached its peak levels at 24 hours and showed a greater level of expression in the bilateral group than the unilateral group at this time point ($p < 0.01$). However, the IL-6 levels did not show a reduced response between the two experimental groups at 6 hours postinjury but did show a very large decrease in its expression in the bilateral group at 12 hours, after which the levels fell back to near baseline. All three cytokines had differing levels of induction relative to their baseline with IL-6 showing the maximal fold change in expression at ~200 to 300 fold, IL-1 β showing 15- to 40-fold change, and TNF- α showing the least change at ~twofold.

Serum IL-6 Levels in Response to Femur Fracture

The rate and amplitude of changes in plasma levels of the APPs are believed to reflect the induction of their synthesis and excretion by various cytokines, such as IL-1 β , TNF- α , and IL-6. In particular, the latter cytokine has been shown to regulate the entire set of APPs. IL-6 increases the expression of genes coding for the positive APPs and down-regulates that of the negative APP.^{9,12} The relatively isolated injury of a femur fracture has profound systemic effects,¹⁶ and levels of IL-6 are believed to correlate with the severity of injury in human trauma patients.¹⁷ To determine a possible role for IL-6 in the molecular events at the fracture site and in the liver after injury, serum IL-6 levels were studied after fracture.

Both UFF and BFF had a similar pattern of response. Both reached their peak in serum IL-6 levels at 6 hours after injury, dropping to near control levels at 24 hours and 48 hours after injury. However, at the 6-hour time-point, serum IL-6 level was significantly higher in the bilateral fracture animals compared with both control and unilateral fracture animals ($p < 0.01$). In more severe injury, increased IL-6 levels persisted at 12 hours before dropping to near control levels at 24 hours and 48 hours after injury. UFF IL-6 achieved lower serum levels overall, but the increased levels were more sustained, remaining increased even at 24 hours (Fig. 4). These serum results are in contrast to the pattern of mRNA expression for IL-6 at the fracture site, where unilateral fracture had a similar level as bilateral fracture in IL-6 mRNA expression at 6 hours rising to twice the level of bilateral fracture at 12 hours before falling to control levels at 24 hours and 48 hours after injury (Fig. 3, B). Serum IL-1 β and TNF- α were not reproducibly detectable by enzyme-linked immunosorbent assay at the time points examined in either UFF or BFF animals (data not shown).

DISCUSSION

We hypothesized that intensity of injury has an effect on the induction, magnitude, and resolution of the APR. Furthermore, we postulated that the level of injury intensity may be a

regulatory mechanism for the ebb and flow phases of the injury response. In this regard, a clear pattern of response to injury magnitude was observed in these experiments. With greater injury, one would expect a greater and faster response. Although we found that increased intensity of injury resulted in a marked alteration in the kinetics and magnitude of the overall response, increased injury magnitude led to a significant delayed induction of the liver's response and an inhibition of the magnitude of the response at the fracture site. In contrast to these results, serum IL-6 levels showed a response that was in a direct association with changes in the magnitude of injury.

Two regional molecular responses were analyzed after femur fracture injury—one local, i.e., the femur fracture wound site, and one distant from the injury site, i.e., the liver. Despite the differences between these two sites and the target genes looked at, there were similarities in the patterns of response to injury magnitude, for both fibrinogen- γ and SAA, and an increased intensity of injury led to a delayed induction of the response seen in the liver (Fig. 2). For cytokine gene expression, at the fracture site, a decrease in magnitude of the response was seen (Fig. 3). Because both responding sites have similar patterns of response to changes in injury intensity, one could argue for a common signaling pathway that affects both responding tissues in a similar way or a common molecular response to injury intensity that allows the overall system to have a graded response to injury, which is manifested in the systemic physiologic response observed as the ebb and flow phase of the injury response.¹⁸

One possible explanation for the changes in the injury response with increased injury would be that with increased intensity of injury, the extent of disruption of normal cellular functions is greater and the organism simply takes a longer time to gather machinery and resources to mount a response leading to a delay. This could explain the results seen in the liver with increased injury leading to a delay in onset and prolonged response but would not so readily explain the results seen at the fracture sites of a generalized and sustained decrease in the response. Another explanation could be that any injury that leads to a set of molecular signaling cascades that are both proinflammatory and counter inflammatory at the same time and increased injury intensity or an increase in injury signaling leads to a change in the balance between these two counter-regulating cascades.¹⁹ This would suggest that systemic effectors play a primary role in modulating the local innate response. The balance between the two competing signaling cascades leads to the systemic and molecular events that we observed. The observation that increased injury intensity leads to a delay in induction, and a modulation of intensity of the responding tissues would make a case for an injury response system, where the downregulating or counter-regulating arm of the response that is systemic in nature becomes dominant as the extent of injury increases. After significant injury, there is an initial period of unresponsiveness, whose duration and extent are dependent on the degree of the initial insult. This is reflected by the delay in the increase in APP in the liver and decreased production of local cytokine gene expression at the fracture site. Such an explanation could be a molecular basis for the clinical manifestation of the “ebb phase” as described by Cuthbertson⁴ in which injured organisms initially respond to injury by a period characterized by hypometabolism; poor tissue perfusion; mild protein catabolism; and low energy expenditure. Later, and enhanced by resuscitation, injured individuals enter the flow phase characterized by hypermetabolism; normal tissue perfusion; increased protein catabolism; and increased energy expenditure. The classic “ebb and flow” injury response raises the question that the differences between unilateral and bilateral fracture may result from the alteration in perfusion; one would expect that bilateral fractured animals would have more blood loss, lower circulating blood volume, and more hypoperfusion than in the unilateral animals. Although we are unable to totally exclude this possibility, extensive differences in organ perfusion seem unlikely given that there was no mortality and no significant gross changes observed when the tissues were harvested between two models. However, microvascular differences in our injury models may very

well play a critical role on local and liver transcriptional events. In other severely injury models, loss of heterogeneity in flow to the liver has been described in femur fracture, cecal ligation/puncture, and a sequential stress combination of both;²⁰ and in another study by Schirmer et al.,²¹ soft tissue injury in addition to induced femur fracture was shown to result in deficits in hepatic perfusion. Therefore, a direct correlation of transcription events with injury-induced changes in local and systemic perfusion needs to be further investigated.

The discordant levels of IL-6 that were seen at the fracture site relative to those that were seen in the serum in relationship to the extent of injury may reflect the choice of time points for IL-6 measurements. Thus, there may be a lag between mRNA expression seen in the tissues and the rise of protein levels that are released in the serum. Alternatively, IL-6 protein levels may have very slow rates of turnover in the serum relative to the mRNAs at the tissue level. However, a second and more intriguing possibility is that the source of serum IL-6 is not from the fracture site and the mRNAs reflect changes that are strictly a local event. The serum IL-6 levels and their response to injury severity may represent a separate compartment and are perhaps the proximal signaling mechanism by which the liver and fracture sites' responses are regulated, i.e., increase in injury severity leads to an early graded increase in serum IL-6 levels that directly or indirectly downregulate the local responses. At this time, one can only speculate as to the source of the serum IL-6 levels, but the spleen or resident macrophages in the liver are possible candidates; if the above is true, it would seem that this counter-inflammatory or counter-regulatory pathway is dominant during the early time points after injury acting potentially as a negative feedback or an inhibitory mechanism that downregulates the local response. This would be consistent with the clinical observations of the dynamics of the ebb and flow phases of injury.¹⁸ The observation that the kinetics of serum IL-6 levels, where in conformity with the extent or degree of injury, would imply a different set of regulatory signal pathways or mechanisms controlling these responses in comparison with those regulating the molecular events in the liver and at the fracture site. This observation may explain the positive effects that can be seen when agents that alter proinflammatory effectors, such as antibodies to proinflammatory cytokines, work best when given before injury occurs and why they have had little or no effect on the overall injury response and outcomes in clinical practice because the molecular signaling cascades regulating the injury response have already been set in motion.

Changes in serum protein concentrations during the APR largely result from alterations in synthesis, secretion, and consumption in response to injury-induced signaling. Relative concentrations of plasma APPs may very well correlate with serum cytokine changes but most likely would be seen after changes in liver mRNA for those proteins are observed. Although we did not look at serum APP levels as that was not the focus of this study. Our data suggest that injury magnitude may regulate transcriptional events in variable locations in a similar manner, and these regulatory processes likely effect serum APP concentrations as well.

In conclusion, we tested the hypotheses that increased intensity of injury would affect the induction, resolution, and duration of the liver's APR; in addition, we tested the hypotheses that injury intensity would alter the rate of proinflammatory cytokine gene expression at the wound site. Our results showed that, indeed, increased injury intensity resulted in an alteration in a tissue response, but these alterations are counterintuitive. Greater injury leads to a delayed induction and prolonged duration of the liver's APR and a decrease in the magnitude of the cytokine responses at the fracture site. These observations could represent a mechanism by which the "ebb" and "flow" phases of injury are regulated. It is important to understand the mechanisms by which the APR is switched on and off as both underresponse

and overresponse and excessively prolonged reaction have proven to be clinically detrimental in trauma patients.

Acknowledgments

Supported by NIH grant (3R01DK064945).

REFERENCES

1. Cerra FB. The systemic septic response: concepts of pathogenesis. *J Trauma*. 1990; 30(12 Suppl):S169–S174. [PubMed: 2254978]
2. Beal AL, Cerra FB. Multiple organ failure syndrome in the 1990s. Systemic inflammatory response and organ dysfunction. *JAMA*. 1994; 271:226–233. [PubMed: 8080494]
3. Baumann H, Gauldie J. The acute phase response. *Immunol Today*. 1994; 15:74–80. [PubMed: 7512342]
4. Cuthbertson DP. The disturbance of metabolism produced by bony and non-bony injury, with notes on certain abnormal conditions of bone. *Biochem J*. 1930; 24:1244–1263. [PubMed: 16744448]
5. Howard JM. Studies of the absorption and metabolism of glucose following injury; the systemic response to injury. *Ann Surg*. 1955; 141:321–326. [PubMed: 14350571]
6. Allison SP, Hinton P, Chamberlain MJ. Intravenous glucose-tolerance, insulin, and free-fatty-acid levels in burned patients. *Lancet*. 1968; 2:1113–1116. [PubMed: 4177162]
7. Carey LC, Lowery BD, Cloutier CT. Blood sugar and insulin response of humans in shock. *Ann Surg*. 1970; 172:342–350. [PubMed: 5459093]
8. Hill AG, Hill GL. Metabolic response to severe injury. *Br J Surg*. 1998; 85:884–890. [PubMed: 9692557]
9. Castell JV, Gomez-Lechon MJ, David M, Fabra R, Trullenque R, Heinrich PC. Acute-phase response of human hepatocytes: regulation of acute-phase protein synthesis by interleukin-6. *Hepatology*. 1990; 12:1179–1186. [PubMed: 1699862]
10. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med*. 1999; 340:448–454. [PubMed: 9971870]
11. Gauldie J, Richards C, Harnish D, Lansdorp P, Baumann H. Interferon beta 2/B-cell stimulatory factor type 2 shares identity with monocyte-derived hepatocyte-stimulating factor and regulates the major acute phase protein response in liver cells. *Proc Natl Acad Sci USA*. 1987; 84:7251–7255. [PubMed: 2444978]
12. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. *Biochem J*. 1990; 265:621–636. [PubMed: 1689567]
13. Bonnarens F, Einhorn TA. Production of a standard closed fracture in laboratory animal bone. *J Orthop Res*. 1984; 2:97–101. [PubMed: 6491805]
14. Wang Z, Burke PA. Effects of hepatocyte nuclear factor-4alpha on the regulation of the hepatic acute phase response. *J Mol Biol*. 2007; 371:323–335. [PubMed: 17574576]
15. Bais M, McLean J, Sebastiani P, et al. Transcriptional analysis of fracture healing and the induction of embryonic stem cell-related genes. *PLoS One*. 2009; 4:e5393. [PubMed: 19415118]
16. Kobbe P, Vodovotz Y, Kaczorowski DJ, Mollen KP, Billiar TR, Pape HC. Patterns of cytokine release and evolution of remote organ dysfunction after bilateral femur fracture. *Shock*. 2008; 30:43–47. [PubMed: 18562923]
17. Biffl WL, Moore EE, Moore FA, Peterson VM. Interleukin-6 in the injured patient. Marker of injury or mediator of inflammation? *Ann Surg*. 1996; 224:647–664. [PubMed: 8916880]
18. Tredget EE, Yu YM. The metabolic effects of thermal injury. *World J Surg*. 1992; 16:68–79. [PubMed: 1290269]
19. Jeschke MG. The hepatic response to thermal injury: is the liver important for postburn outcomes? *Mol Med*. 2009; 15:337–351. [PubMed: 19603107]

20. Kamoun WS, Shin MC, Keller S, Karaa A, Huynh T, Clemens MG. Induction of biphasic changes in perfusion heterogeneity of rat liver after sequential stress in vivo. *Shock*. 2005; 24:324–331. [PubMed: 16205316]
21. Schirmer WJ, Schirmer JM, Townsend MC, Fry DE. Femur fracture with associated soft-tissue injury produces hepatic ischemia. Possible cause of hepatic dysfunction. *Arch Surg*. 1988; 123:412–415. [PubMed: 3348730]

Figure 1. Mouse femur fracture and fixation. Postprocedure of femur fracture. The *arrow* indicates that X-ray image reveals a right-sided femur fracture and the location of the intramedullary pin.

Figure 2.

The expression of APR genes in the liver tissue in response to fracture. The mRNA levels of fibrinogen- γ (A) and SAA-3 (B) were shown after UFF and BFF injury. Data shown represent mean $\pm$ SE from three to four separate animals. * $p < 0.0003$ versus control (0 time point); # $p < 0.02$ indicates a significant difference between UFF and BFF. ◆ $p < 0.04$ versus control (0 time point).

Figure 3.

The expression of cytokines in the femur fracture site. The mRNA expression levels of IL-1 β (A), IL-6 (B), and TNF- α (C) at the fracture site were illustrated in UFF and BFF animals. Data shown represent mean $\pm$ SD from three to four separate animals. * $p < 0.05$ and ** $p < 0.01$ versus control (0 time point) and † $p < 0.05$ and ‡ $p < 0.01$ indicate a significant difference between UFF and BFF.

Figure 4.

The serum level of IL-6 in response to fracture. Enzyme-linked immunosorbent assay was used to quantify levels of IL-6 in the serum after injury procedure. The kinetics of IL-6 serum levels were shown after a UFF and BFF injury. Data shown represent mean $\pm$ SE from three to four separate animals. ** $p < 0.01$ versus control (0 time point) and ‡ $p < 0.01$ indicate a significant difference between UFF and BFF.