Elevated leptin expression in rat model of traumatic spinal cord injury and femoral fracture

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Background: Few studies have reported a relationship between leptin induced by spinal cord injury (SCI) and healing bone tissue.

Objective: To observe serum and callus leptin expression within the setting of fracture and traumatic SCI.

Methods: Seventy-two male Sprague Dawley rats were randomized equally into four groups: control, SCI group, fracture group, and fracture/SCI group. Rats were sacrificed at 7, 14, 21, and 28 days post-fracture/SCI. Serum leptin was detected using radioimmunoassay at 1, 7, 14, 21, and 28 days, and callus formation was measured radiologically at 14, 21, and 28 days. Callus leptin was analyzed by means of immunohistochemistry.

Results: Serum leptin in the fracture group, SCI group, and combined fracture/SCI group were all significantly increased compared to control group at the 1, 7, 14, and 28-day time points ($P < 0.05$). Serum leptin in the combined fracture/SCI group was significantly higher than in the fracture group at 7, 14, and 21 days ($P < 0.05$), and higher than in SCI groups at 14 and 21 days after operation ($P < 0.05$). The percentage of leptin-positive cells in the fracture/SCI callus, and callus volume was significantly higher than in the fracture-only group ($P < 0.001$).

Conclusions: Overall, elevated leptin expression was demonstrated within healing bone especially in the 21 days of a rat model combining fracture and SCI. A close association exists between leptin levels and the degree of callus formation in fractures.

Keywords: Leptin, Fracture, Osteogenesis, Spinal cord injuries, Bone, Callus

Introduction

Clinical and observational studies have shown enhanced osteogenesis in patients sustaining limb fracture with associated spinal cord injury (SCI).¹ Accelerated fracture healing and heterotopic ossification are well-known phenomena in these patients. Recent work with the serum of patients with SCI seems to indicate that humoral mechanisms may be involved.² These include predominantly serum factors such as growth hormone, transforming growth factor-beta, nerve growth factor, insulin-like growth factor II, platelet-derived growth factor, and interleukin-1 (IL-1) and IL-6.^{3,4} It is, therefore, possible that bone resorption and collagen degradation in acute SCI may well result in the release of osteo-inductive factors.⁵ With the recent finding that bone formation is regulated by leptin through the

hypothalamus and the sympathetic nervous system *in vitro* and *in vivo*,^{6,7} it is distinctly possible that the disruption of this pathway is a key factor in osteogenesis within the context of SCI. But very few studies have been reported on the changes in serum leptin induced by SCI.

The aim of this study was to measure changes in serum leptin after SCI and femoral fracture and to observe any association between leptin levels and the degree of fracture healing. In addition, this study will form comparisons between the healing response in SCI rats and those without spinal injury in order to quantify the effect of SCI on bone formation. A related focus will be the temporal relationship between leptin levels and the quantity of callus formation after SCI.

Understanding the role of leptin within the context of bone metabolism and injury may provide further avenues for the treatment of both non-union and heterotopic bone formation.

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Materials and methods

Ethical approval for this study was granted by the ethical board of The Affiliated People's Hospital with Jiang-su University, China. Adult male Sprague Dawley rats ($n = 72$, 250–290 g, average weight = 267 ± 17.5 g, purchased from Shanghai Slaccas Laboratory Animal Co., Ltd, Shanghai, P.R. China) were maintained at $23 \pm 1^\circ\text{C}$ on a 12-hour light/12-hour dark cycle and all animals were housed individually in specific pathogen-free conditions with unlimited access to water and laboratory food and were treated strictly in accordance with institutional ethical guidelines. A rat leptin kit (LINCO Corporation, St Louis, MO, USA) using a mouse monoclonal anti-myosin-Leptin (Sigma-Aldrich Inc., St Louis, MO, USA) was used for the radioimmunoassay. Rats were randomized into four groups: unoperated control, SCI group, fracture group, and fracture/SCI group ($n = 18$ per group). The 2-ml blood samples were collected from each rat at approximately 9 a.m. at 1, 7, 14, 21, and 28 days and these blood samples were centrifuged at 4°C for 30 minutes at 4000 rpm, and serum aliquots were frozen at -20° . Rats were sacrificed by overdose with sodium pentobarbital (100 mg/kg, intraperitoneally (i.p.)), perfused transcardially with 0.9% NaCl and 10% neutral formalin at 14, 21, and 28 days, respectively, after operation.

Radioimmunoassay

Leptin concentration in serum was detected using radioimmunoassay. In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with dilution of ^{125}I -labeled rat leptin antiserum such that the concentration of antigen-binding sites on the antibody is limited. If unlabeled antigen (testing rat serum) is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited antibody. Then antibody-bound tracer can be separated from unbound tracer, and finally, an instrument (DFM-96 radioimmunity-counter, Nanjing Medical University, China) to count radioactivity and quantify leptin concentration. Results are in ng/ml. Callus formation was monitored with radiographs and callus volumetric analysis was performed. Leptin expression in callus was also analyzed via immunohistochemistry.

SCI model

Animals were sedated with 1 mg/kg ketamine intramuscularly (i.m.), and then were deeply anesthetized with 1.5–2.5% isoflurane. After midline cervical skin incisions, the caudal half of the C5 dorsal lamina and

the entire C6 dorsal lamina were removed, thereby exposing the C7 spinal cord segment. The dura was slit longitudinally along the midline and gently retracted. An iridectomy micro-knife with blade rotated 90° to the long axis of the spinal cord was mounted on a stereotaxic arm, and positioned 0.5 mm to the left of the spinal cord midline, aiming to completely transect the right lateral hemicord. The blade was lowered into the spinal cord completely from the dorsal to the ventral surface to place an initial central incision, thereby separating the left and right halves of the cord. This initial cut established the medial-most position of the lesion. Then, under the visual guidance of the operating microscope and using long iridectomy scissors, the lesion of the right hemicord was completed (Figs 1A and D). No operations were performed on control rats. SCI rats received daily assistance in bladder emptying until spontaneous miction recovered. All rats were fed with commercial rat chow and housed in a controlled environment at 22°C with a 12-hour light/dark cycle. The SCI and SCI/fracture total mortality rate was 13.1%. When a rat died, we performed the operation on an additional rat. The average body weights of the rats in all of the groups at the 1, 7, 14, 21, and 28 days were 262 ± 12.2 , 255 ± 16.5 , 265 ± 13.7 , 271 ± 20.4 , 272 ± 18.9 g, and analysis of variance (ANOVA) did not detect a significant difference in these average weights. The Basso, Beattie, and Bresnahan (BBB) locomotor scale method⁸ was used to evaluate post-operative functions for SCI and SCI/fracture groups (Table 1).

Femoral fracture model

Rats were sedated with 1 mg/kg ketamine i.m., and then were deeply anesthetized with 1.5–2.5% isoflurane. Femoral osteotomy and fixation were performed in the same manner as previously reported.⁹ Briefly, a transverse osteotomy was made at the midshaft of the right femur and intramedullary fixation was performed using a stainless-steel wire (diameter, 1.5 mm). The fracture fragments were reduced and stabilized. Wires were cut on the surface of the intercondylar groove to avoid restriction of motion of the knee joint. All rats were fed commercial rat chow, and housed in cages at 22°C with a 12-hour light/dark cycle. Unrestricted activity was allowed after recovery from anesthesia.

Tissue preparation and histological analysis

Rats were sacrificed by overdose of sodium pentobarbital (100 mg/kg, i.p.), perfused transcardially with 0.9% NaCl and 10% neutral formalin. After sacrifice, fractured femurs from rats were dissected and carefully cleaned of

Figure 1 Photomicrographs showing the pathological changes including bleeding and edema 1 day after operation ($\times 200$) (A). Normal graph ($\times 200$) (B). Transmission electron microscope reveals disordered axonal structure, edema, and myelinolysis 1 day after operation ($\times 5000$) (C). Normal ultrastructure of the spinal cord in the control group ($\times 5000$) (D).

muscle around the fracture callus to preserve callus integrity. Calluses from 14, 21, and 28 days after fracture were then fixed in 4% paraformaldehyde, the intramedullary wires were removed, and the specimens were decalcified before paraffin embedding. Sagittal sections ($5\text{--}7\text{ }\mu\text{m}$) were made through the fracture site and mounted on Fisherbrand Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Tissue sections were deparaffinized with xylene, and then rehydrated through a series of decreasing ethanol concentrations. Sections were stained with hematoxylin–eosin for histological evaluation of healing at 14, 21, and 28 days after fracture.

Assay for callus proliferation

Perkins volume formula¹⁰ was used as a measure of volume of the fracture callus of experimental rats and control rats at 14, 21, and 28 days after fracture. Anterior–posterior radiographs of fractures were taken of all rats and the volume of callus was calculated

using the Perkins volume formula: $2\pi R1(R2-R1)L$ (where $R1$ = femur radius; $R2$ = callus radius; L = length of callus).

Immunohistochemistry localization of leptin

Sections were washed in phosphate-buffered saline with 0.1% Triton X-100 (Sigma Diagnostics, St Louis, MO, USA) and endogenous peroxidase was blocked using a solution of 30% methanol and 3% H_2O_2 for 30 minutes. The tissue sections were pre-treated with 10% normal rabbit serum diluted in phosphate-buffered saline (blocking buffer) for 30 minutes. The primary antibody was rat anti-human leptin monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA), which was diluted 1:200 in blocking buffer. Sections were incubated in diluted primary antibody for 1 hour at room temperature and then washed three times with phosphate-buffered saline with 0.1% Triton X-100 for 5 minutes each. The secondary antibody used was mouse anti-rat

Table 1 BBB scores of four groups at different time points after SCI ($\bar{X} \pm S$) $N = 6$

Group	Time point after injured				
	1 day	7 days	24 days	21 days	28 days
Control group	20.50 ± 0.55	20.50 ± 0.55	20.67 ± 0.52	20.83 ± 0.41	20.67 ± 0.52
Fracture group	19.16 ± 0.75	19.50 ± 0.84	20.17 ± 1.17	20.33 ± 1.11	20.16 ± 1.02
SCI group	$1.16 \pm 0.75^*$	$1.50 \pm 0.84^*$	$6.17 \pm 1.17^*$	$10.33 \pm 1.11^*$	$14.16 \pm 1.16^*$
Fracture + SCI	$1.17 \pm 0.25^{***}$	$1.33 \pm 0.52^{***}$	$4.16 \pm 0.75^{***}$	$8.17 \pm 0.75^{***}$	$11.17 \pm 0.98^{***}$

*Showed to compare with the control group, $P < 0.05$.

**Showed to compare with the SCI group, $P > 0.05$.

polyclonal antibody (R&D Systems, Minneapolis, MN, USA) diluted 1:1000 in blocking buffer. After three washes, the tissue sections were incubated in diluted secondary antibody for 30 minutes at room temperature and then washed three times in phosphate-buffered saline with 0.1% Triton X-100 for 5 minutes each. They were then rinsed and reacted in the dark with streptavidin-peroxidase reagent. Finally, an immunoreactive product was visualized. These sections were washed with water, counterstained with hematoxylin, dehydrated, cleared with 100% xylene, and mounted for microscopic examination with an Olympus BH-2 microscope (Olympus Corporation of America, New Hyde Park, NY, USA). Negative controls were stained as above but without primary antibody.

Leptin expression analysis

Leptin staining was assessed in eight representative 4- μ m sections, which were chosen blindly and randomly from the middle of each sampled callus and the distance between each section was 5 μ m. Cells with light-brown staining particles in the cytoplasm were regarded as positive. Positive cells were counted at $\times 400$ magnification, each field equating to an area of approximately 100 μ m $\times$ 100 μ m. The count was performed in four random choice fields for each tissue section, leptin-positive cells were counted in callus in blinded fashion. A stereological optical fractionator technique was used to provide an unbiased estimate of the number of cells as described previously.¹¹ Then an average number of leptin-positive cells were obtained.

Average leptin-positive cell counts were normalized to the volume of callus by dividing the number of leptin-positive cells by the callus volume (calculated with the Perkins formula). The normalized result was then expressed as a number of positive cells per mm³.

Statistics

We used the SPSS 13.0 statistical analysis software package for data analysis. Mean and standard deviation of the mean of positively stained cells, callus volume, and the serum level of leptin were evaluated. One-way

ANOVA and Student–Newman–Keuls tests were used in this study. *P* values 0.05 were considered statistically significant. Linear regression analyses were performed between leptin concentration and volume of callus formation in the fracture/SCI group.

Results

Serum levels of leptin

The serum levels of leptin in the SCI group, fracture group, and fracture/SCI group were significantly increased compared with that in the control group from 1 to 21 days after operation ($P < 0.05$) (Table 2; Fig. 2). The rate of increase in concentration of serum leptin in the SCI group and fracture/SCI group was more rapid than in other treatment groups at 7 days post-operation ($P < 0.05$), but ANOVA did not detect a significant difference between the SCI and fracture/SCI groups ($P > 0.05$). The concentration of serum leptin in the fracture/SCI group continued to be significantly greater than in other treatment groups at 14, 21 days post-operation ($P < 0.001$). This difference was not statistically significant between fracture/SCI and SCI groups at 28 days.

Figure 2 Serum leptin concentrations in from days 1, 7, 14, 21, and 28 in the four cohorts.

Table 2 Changes in serum levels of leptin in different phases in four groups. Unit: ng/ml ($\bar{X} \pm S$), $N = 6$

Groups	1 day	7 days	14 days	21 days	28 days
Control group	1.25 ± 0.06	1.28 ± 0.03	1.33 ± 0.07**	1.40 ± 0.11**	1.32 ± 0.06
Fracture group	2.67 ± 0.10*	2.67 ± 0.07*	2.31 ± 0.13***	2.20 ± 0.09***	1.85 ± 0.09
SCI group	2.53 ± 0.14*	3.54 ± 0.13***	3.23 ± 0.09***	2.84 ± 0.18***	2.47 ± 0.15
Fracture + SCI	3.30 ± 0.15*	3.81 ± 0.11***	4.53 ± 0.11*	3.55 ± 0.18*	2.86 ± 0.16

* $P < 0.05$ compared with serum levels of leptin in control group at 1, 7, 14, 21 days.

** $P < 0.001$ compared with serum levels of leptin in fracture + SCI group at 14, 21 days.

*** $P > 0.05$ ANOVA did not detect a significant difference in SCI group and fracture/SCI group.

Figure 3 Volume of callus from days 14, 21, and 28 in fracture and fracture/SCI groups. At 21 and 28 days, the volume of callus in fracture/SCI group are significantly higher than in the fracture-only group ($P < 0.001$).

Radiological analysis

At time-point 14 days after operation, evidence of callus formation was found at the fracture site in the fracture/SCI group, whereas callus formation in the fracture-only group was less than in the fracture/SCI group. This difference, however, did not reach statistical significance ($P > 0.05$) (Table 3; Figs 4A and B).

At time-point 21 days after operation, callus formation was more rapid in the fracture/SCI group and the volume of callus formation in the fracture/SCI group was found to be significantly greater than in the fracture-only group ($P < 0.001$) (Table 3; Figs 4C and D). At 28 days, the volume of callus in the fracture/SCI group continued to be significantly larger than in the fracture group ($P < 0.001$).

Histomorphological observation

The histological appearance of the fracture callus was different between the fracture group and fracture/SCI group. At the 14-day time point, fracture callus from the fracture/SCI group showed a well-organized callus with areas of newly formed fibrous callus, calcified cartilage, chondrocytes, and hypertrophic chondrocytes. At time-points 21 and 28 days, the fracture/SCI group demonstrated more mature trabecular bone formation

and larger external callus compared to the fracture-only group (Figs 5A and B).

Immunohistochemical analysis

Throughout the process of fracture healing, products of leptin can be detected immunohistochemically in many cells around the fracture site including osteoblast, fibroblast, mesenchymal, and cartilage cells.

At 14 days after operation, a greater percentage of leptin-positive cells were found in the fracture/SCI group compared to the fracture-only group; however, this did not reach statistical significance (Table 4). For time-points 21 and 28 days, the percentage of positive cells (percentage of positive cells is equal to number of positive cells divided by number of osteoblast, fibroblast, mesenchymal, and cartilage cells) in callus for the fracture/SCI group was significantly higher than that of the fracture-alone group ($P < 0.05$) (Table 4; Figs 5C and D). When analyzing leptin-positive cells normalized to callus volume, the fracture/SCI group demonstrated a higher result at all time points, but there was statistical significant difference only at 21 and 28 days (Table 5).

Discussion

Leptin, the circulating protein product of the Ob gene, was initially discovered as a satiety factor regulating food intake and energy expenditure.¹² It is produced primarily by adipocytes and acts on the hypothalamus, but important non-adipocytic, extra-hypothalamic pathways also exists.^{13,14} More recently, the role of leptin in bone metabolism has been identified¹⁵ and is undergoing intensive characterization, especially in the context of trauma and osteoporosis.

By acting on the hypothalamus and increasing both sympathetic output and β_2 -adrenergic receptors on the surface of osteoblasts, leptin exerts an anti-osteogenic effect.⁶ Peripherally acting leptin, however, has an opposite effect by promoting bone mineralization¹⁶ and osteoblast to osteocyte differentiation.^{17,18} Some studies suggest that leptin increases the proliferation and mineralization of osteoblasts and inhibits apoptosis of osteoblasts.¹⁹ Furthermore, both human and rat osteoblasts have been reported to express functional

Table 3 Volume of fracture callus in fracture and fracture + SCI groups. Unit: mm³ ($\bar{X} \pm S$)

Groups	Numbers	14 days	21 days	28 days
Fracture group	6	37.12 $\pm$ 2.86	50.23 $\pm$ 7.19*	67.59 $\pm$ 7.55**
Fracture + SCI	6	40.05 $\pm$ 2.30	62.74 $\pm$ 9.65	89.42 $\pm$ 14.46

* $P < 0.001$ compared with volume of callus in fracture + SCI group at 21 days.

** $P < 0.001$ compared with volume of callus in fracture + SCI group at 28 days.

Figure 4 Radiographs of fractured femoral in the fracture group and fracture/SCI groups. At 14 days after the fracture, the fracture line is visible and a small amount of callus is visible in both groups (white arrow). The callus became dense and callus formation at the fracture site in fracture/SCI group (B) occurred more rapidly than in the fracture group (A) at 21 days. Volume of callus in the former group was significant larger than in the latter group. At 28 days the size of callus in both groups was increased, with fracture/SCI group (D) again significantly larger than in the fracture-only group (C).

ObR isoforms,²⁰ suggesting that leptin may have local effects on bone. On the contrary, leptin deficiency or insensitivity, resulting from either ob/ob or db/db, is associated with a profound negative effect on biomechanical strength in mouse femurs.²¹ In the physiological resting state, these two opposing actions of leptin are in balance.²²

Leptin mediates many of its physiological effects by activating neurons in the hypothalamus that innervate and stimulate sympathetic pre-ganglionic neurons in the spinal cord. The stress response to trauma is characterized by sympathetic activation and influenced secretion of pituitary hormones.²³ The adrenergic response is related to the extent of trauma and is determined by afferent signals (nociceptive pathways and humoral mediators) originating from the site of injury.

Linked in with this is the stress response of the neuro-endocrine system where several cytokines and hormonal factors such as insulin and glucocorticoids have been shown to alter serum leptin levels.^{24–28} SCI increases levels of proinflammatory cytokines and chemokines and activates a variety of endogenous inflammatory cells including microglia and astrocytes.^{29–31} These parenchymal cells contribute to an increase in endogenous proinflammatory modulators that can promote cellular responses to trauma and contribute to neuronal death. In particular, the proinflammatory cytokine IL-1 is induced rapidly after SCI.³² Notably, IL-1, a key cytokine in tissue injury and inflammation is found to increase leptin levels³³ and this inflammatory pathway is likely to be involved in our findings of raised leptin in trauma.

Figure 5 At time-point 21 days, the fracture/SCI group (B) demonstrated more mature trabecular bone formation (black arrows) and larger external callus compared to the fracture-only group, which showed a large amount of formed fibrous callus (black arrows) (A). Sections were stained with hematoxylin–eosin. Magnifications were $\times 100$ (A/B). Immunohistochemical staining for leptin (black arrows) in the fracture group (C) and the SCI/fracture group at 21 days (D). Note the general increased amount of leptin staining in the SCI/fracture group for these time points. Magnifications were $\times 400$ (C/D).

Table 4 The mean percentage of leptin-positive cells in callus of fracture and fracture + SCI groups. Unit: % ($\bar{X} \pm S$), $N = 6$

Group	14 seconds	21 days	28 days
Fracture group	6.7 ± 1.65	$11.75 \pm 2.70^*$	$5.25 \pm 1.88^{**}$
Fracture + SCI	9.35 ± 3.02	19.25 ± 4.35	8.75 ± 3.17

* $P < 0.05$.

** $P < 0.05$ compared with the mean percentage of positive cells in callus of fracture + SCI group at 21, 28 days.

Table 5 Number of leptin-positive cells per unit callus volume (lep.c/ca.v), $N = 6$

Group	14 days	21 days	28 days
Fracture-only	0.13 ± 0.02	$0.15 \pm 0.01^*$	$0.20 \pm 0.03^*$
Fracture/SCI	0.16 ± 0.03	0.19 ± 0.03	0.26 ± 0.02

* $P < 0.05$; compared with the lep.c/ca.v of fracture/SCI group at 21 and 28 days.

Serum leptin in the SCI group, fracture group, and fracture/SCI group was significantly increased compared to the control group from 1 day after operation ($P < 0.05$) (Fig. 2). With regard to elevated leptin levels in the fracture-only condition, there are several explanations. First, the hypermetabolic response from injury generally results in mobilization of more free fatty acids, which leads to leptin elevation by

neuroendocrine feedback regulation.³⁴ Second, although not specifically documented in our study, a pulmonary inflammatory response common in skeletal trauma, like adult respiratory distress syndrome, will lead to hypoxia, which augments adipocyte expression of leptin.³⁵ Third, affluent medulla ossium flava are released from the broken ends of fractured bone, and because this bone marrow is composed of mainly adipose-degenerated hematopoietic cells, there is a local induction of leptin delivery.³⁶ At 14 days after operation, along with the fracture of the stable, serum leptin also decreased.

Rats with SCI experience dysfunction of the sympathetic nervous system, which results in an altered hormonal and metabolic response. After SCI, the direct damage, including diffuse axonal injury, contusions, intracranial hematoma, and pathophysiology, is amplified by secondary or delayed events including hypoxia, hypotension, ischemia, edema, increased intracranial pressure, all of which lead to hypothalamic–pituitary–adrenal (HPA) axis damage and dysfunction.³⁷ Local sympathetic denervation of white adipose tissue in rats induces pre-adipocyte proliferation. Furthermore, adrenergic stimulation down-regulates the leptin expression and secretion by adipocyte.²⁶ Sympathetic denervation of the adrenal glands causes reduced adrenal

corticosteroid response to stress and stimuli.³⁸ These alterations may also indirectly play a role in the elevated serum leptin level.

An interesting finding in the fracture/SCI group, which mimics the injuries and recovery response of the multi-trauma patient, was a significantly higher leptin level than other treatment groups for time-points 14 and 21 days (Fig. 2; Table 2). The volume of callus and the percentage of leptin-positive cells in the fracture/SCI group was also significantly greater than in the fracture-only group at 21 and 28 days (Figs 3–5; Tables 3 and 4). These results support the concept that impaired HPA axis may result in additional, peripheral secretion of leptin and bone regeneration through loss of sympathetic inhibitory control.^{39–41} SCI is more likely to result in increased sympathetic outflow in the acute period, but it is evident from our data that over time, the peripheral effect of increasing leptin may outweigh any potential sympathetic inhibition of leptin and bone formation.

Elevated serum leptin may then act peripherally to induce myeloid precursor cell differentiation, osteoblast proliferation, and accelerate the mineralization of bone at the fracture site.^{16–18} As previously reported,⁴² leptin might also enhance alkaline phosphatase activity, secretion of osteocalcin and expression of type I collagen mRNA. All these factors, which are involved in *de novo* bone formation, will contribute to enhanced callus formation post-injury.

The peak in callus formation demonstrated at 28 days (Fig. 3; Table 3), corresponds with the known process of callus resorption, most likely by osteoclasts, facilitating consolidation and true bony healing.⁴³ Changes in callus volume also influence the normalized leptin-positive cell count (Table 5). In this case, a significant

difference was still noted between fracture-only and fracture-SCI groups but only at the 21- and 28-day time points. Lack of statistical difference at 14 days may be related to fewer positive cells for the fracture-SCI group (Table 4) being offset by smaller callus volume for this group. Hence, it may be misleading to consider the normalized data in isolation.

Furthermore, we also found a positive correlation between leptin concentration in serum and volume of callus formation in the fracture/SCI group (Fig. 6). From 21 days to 28 days, the higher leptin level in the SCI/fracture group was accompanied by a larger amount of callus, which again may suggest that leptin is a mediator to the bone-forming cells.

The present study revealed that activation of endogenous leptin secretion of the organism started immediately after the SCI/fracture, and it showed a greatly increasing trend for three operation groups. These higher leptin levels are associated with increased callus formation in femoral fractures and also increased leptin expression in the fracture callus. Leptin does not reach a maximum level until 14 days after injury. In future studies on leptin in SCI and fracture healing, it will be important to identify the temporal relationships between serum leptin, and physical signs of sympathetic activity. Furthermore, it would be beneficial to perform a study including a leptin-knockout group to more accurately determine the effect of leptin on callus formation in SCI.

Our studies are important because treatment strategies targeting systemic factors may be necessary to promote maximum benefit and outcome in a subset of SCI patients coping with fractures. Increasingly, it is being recognized that a specific population of SCI patients develop heterotopic ossification. This will influence the timing of surgery after SCI/fractures.

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Figure 6 Positive correlation between serum leptin concentration and volume of callus formation in the fracture/SCI group from 14 days to 28 days.

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