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Elevated leptin expression in a rat model of fracture and traumatic brain injury

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Abstract

A few studies have reported a relationship between leptin induced by brain injury and healing of bone tissue. Our objective was to measure serum and callus leptin expression within the setting of fracture and traumatic brain injury (TBI). Sixty-four male Sprague–Dawley rats were randomised equally into four groups: control, TBI group, fracture group and fracture/TBI group. Rats were sacrificed at 2, 4, 8 and 12 weeks after fracture/TBI. Serum leptin was detected using radioimmunoassay, and callus formation was measured radiologically. Callus leptin was analysed with immunohistochemistry. Serum leptin was significantly increased in the fracture, TBI and combined fracture/TBI groups compared with the control group at 2 weeks ($P < 0.05$). Serum leptin was significantly higher in the combined fracture/TBI group than in the fracture and TBI groups at 4 and 8 weeks ($P < 0.05$). The percentage of leptin-positive cells in the callus and callus volume were significantly higher in the fracture/TBI group than in the fracture-only group ($P < 0.001$). Thus, we demonstrated elevated leptin expression within healing bone, particularly in the first 8 weeks of a rat model combining fracture and TBI. A close association exists between leptin levels and the degree of callus formation in fractures.

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Introduction

Clinical and observational studies have shown enhanced osteogenesis in patients who sustain limb fracture at the same time as traumatic brain injury (TBI) (Perkins & Skirving 1987; Spencer 1987). Accelerated fracture healing and heterotopic ossifications are well-known phenomena in these patients. With the recent finding that bone formation is regulated by leptin through the hypothalamus and the sympathetic nervous system (Takeda et al 2002; Bonnet et al 2007), it is distinctly possibly that the disruption of this pathway is a key factor in osteogenesis within the context of TBI, and may therefore provide a focus for future research into the treatment of heterotopic bone and fracture non-union in both TBI and non-TBI patients.

Associations between other bone-associated metabolic factors and this phenomenon of fracture healing in TBI have already been demonstrated. These include predominantly serum factors such as growth hormone, transforming growth factor-β, nerve growth factor, insulin-like growth factor II, platelet-derived growth factor, interleukin (IL)-1 and IL-6 (Pasinetti et al 1993; Kim et al 1996; Rigaux et al 2005). Very few studies have been reported on the changes in serum leptin induced by TBI (Simonsen et al 2007). 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.

The aim of this study was to measure changes in serum leptin after TBI and femoral fracture and observe any association between leptin levels and the degree of fracture healing. A further aim was to compare the healing response in TBI rats and those without cerebral injury in order to quantify the effect of TBI on bone formation. The temporal relationship between leptin levels and callus formation after TBI was also investigated.

Materials and Methods

Experimental rats

Approval for this study was granted by the ethical board of the First Affiliated Hospital with Nanjing Medical University, China.

Adult male Sprague–Dawley rats (n = 64; 250–290 g, purchased from Shanghai Slaccas Laboratory Animal Co. (Shanghai, China) were maintained at 23 ± 1°C with a 12 h light–dark cycle. 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.

Rats were randomised into four groups: unoperated control, TBI group, fracture group and fracture/TBI group (n = 16 per group). Four rats from each group were killed at 2, 4, 8 and 12 weeks after surgery.

Leptin radioimmunoassay

The concentration of leptin in serum was detected using a rat leptin radioimmunoassay kit (Linco Corp., St Charles, MO, USA). This involved incubating a fixed concentration of labelled tracer antigen with a dilution of ^{125}I-labelled rat leptin antiserum (mouse monoclonal anti-myosin-leptin (Sigma-Aldrich Inc., St Louis, MO, USA)), limiting antigen binding sites on the leptin antibodies. Unlabelled antigen (rat testing serum) was added to this system, resulting in competition for binding sites between labelled tracer and the unlabelled leptin antigens. Antibody-bound tracer was separated from unbound tracer. A DFM-96 radioimmunity-counter (Nanjing Medical University, China) was used to count radioactivity and therefore quantify leptin concentration in ng mL^{-1}.

TBI model

Animals were anaesthetised with 2% isoflurane and then given oxygen with 0.75% isoflurane through a fixed face mask on a stereotactic platform. The skull was exposed and a craniectomy was performed laterally, midway between Lambda and Bregma, between the central suture and the left temporal ridge, using a 6 mm trephine. The exposed dura was subjected to a 5 mm-diameter piston impact of 3.0 mm depth, 4 m s^{-1} velocity and 100 ms duration. The scalp was closed without replacement of the bone flap. Anaesthesia was discontinued, and the animal was assessed for exclusion criteria (latency of pinna and corneal reflexes and righting response). No surgery was performed on control rats. The total mortality of the TBI and TBI/fracture groups combined was 15.6%. Rats that died during surgery were replaced and an additional procedure was performed.

Femoral fracture model

Femoral osteotomy and fixation were performed as reported previously (Li et al 2000) during the same procedure as TBI. Briefly, a transverse osteotomy was made at the midshaft of the femur and intramedullary fixation was performed using a stainless steel wire (diameter 1.5 mm). The fracture fragments were reduced and stabilised. The wires were cut on the surface of the intercondylar groove to avoid restricting movement of the knee joint. Unrestricted activity was allowed after recovery from anaesthesia.

Tissue preparation and histologic analysis

After sacrifice, fractured femurs from rats were dissected and carefully cleaned of muscle around the fracture callus to preserve callus integrity. Calluses from weeks 2, 4, 8 and 12 after fracture were then fixed in 4% paraformaldehyde, the intramedullary wire was removed, and the specimen was decalcified before paraffin embedding. Sagittal sections (5–7 μm) were made through the fracture site and mounted on Fisherbrand Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were stained with haematoxylin & eosin for histological evaluation of healing.

Immunohistochemistry localisation of leptin

Tissue sections were deparaffinised with xylene, and then rehydrated through a series of decreasing ethanol concentrations. Sections were washed in phosphate-buffered saline (PBS) 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% hydrogen peroxide for 30 min. The tissue sections were pretreated with 10% normal rabbit serum diluted in PBS (blocking buffer) for 30 min. The primary antibody was rat anti-human leptin monoclonal antibody (Sigma-Aldrich), which was diluted 1:200 in blocking buffer. Sections were incubated in diluted primary antibody for 1 h at room temperature and then washed three times with PBS with 0.1% Triton X-100 for 5 min each. The secondary antibody used was rat anti-mouse 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 min at room temperature and then washed three times in PBS with 0.1% Triton X-100 for 5 min each. Sections were washed with water, counterstained with haematoxylin, dehydrated, cleared with 100% xylene, and mounted for microscopic examination with an Olympus BH-2 microscope (Olympus Corp., New Hyde Park, NY, USA). Negative controls were stained as above but without primary antibody.

Assay for callus proliferation

The Perkins volume formula (Perkins & Skirving 1987) was used as a measure of the volume of the fracture callus of experimental rats and control rats at 2, 4, 8 and 12 weeks after fracture. Anterior–posterior radiographs of all fractures were taken and the volume of callus was calculated using the formula: $2\pi R1(R2 - R1)L$, where R1 = femur radius, R2 = callus radius and L = length of callus.

Leptin expression analysis

Leptin staining was assessed in eight representative 4 μm sections from the middle of each sampled callus. Cells with light-brown staining particles in the cytoplasm were

regarded as leptin-positive. Positive cells were counted at 400× magnification, equating to an area of approximately 100 μm × 100 μm. The count was performed in four fields for each tissue section, and an average number of leptin-positive cells was obtained.

Average leptin-positive cell counts were normalised to the callus volume by dividing the number of leptin-positive cells by the callus volume (calculated using the Perkins formula). The normalised result was then expressed as a number of positive cells per mm^3.

Statistical analysis

We used the SPSS 12.0 (SPSS Inc., Chicago, IL, USA) statistical analysis software package for data analysis. Mean and s.d. of the mean of positively stained cells and the serum level of leptin were evaluated. One-way analysis of variance and the Student–Newman–Keuls test were used. P values below 0.05 were considered statistically significant. Linear regression analysis was performed to assess the relationship between callus volume and leptin concentration in the fracture/TBI group.

Results

Serum leptin levels

Serum leptin levels were increased significantly in the TBI, fracture-only and fracture/TBI groups compared with the control group at 2 weeks post-operation ($P < 0.05$) (Figure 1). The rate of increase in serum leptin concentration was more rapid in the fracture/TBI group than in other treatment groups at 4 weeks post-operation ($P < 0.001$). The concentration of serum leptin in the fracture/TBI group continued to be significantly greater than in other treatment groups at 8 weeks post-operation ($P < 0.05$). This difference was not statistically significant at 12 weeks.

Radiological analysis

At 2 weeks after surgery, evidence of callus formation was found at the fracture site in the fracture/TBI group, whereas callus formation in the fracture-only group was minimal; however, this difference was not statistically significant (Figure 2). At 4 weeks after surgery, callus formation was more rapid in the fracture/TBI group than in the fracture-only group, and the volume of callus formation in the fracture/TBI group was significantly greater ($P < 0.001$) (Figure 2). At 8 weeks, the callus volume in the fracture/TBI group continued to be significantly larger than in the fracture-only group ($P < 0.001$) (Figure 2). Complete fracture healing was found in both the fracture/TBI and fracture-only groups at 12 weeks. The fracture line became invisible, and the fracture calluses were partly absorbed and reduced compared with those at 4 and 8 weeks. There was no significant difference in callus volume between the two groups.

Histomorphological observation

The histological appearance of the fracture callus differed between the fracture-only and fracture/TBI groups. At the 2-week time point, fracture callus from the fracture/TBI group showed a well-organised callus with areas of newly formed fibrous callus, calcified cartilage, chondrocytes and hypertrophic chondrocytes. At the 4- and 8-week time points, calluses in the fracture/TBI group demonstrated more mature trabecular bone formation and larger external callus compared with the fracture-only group. At the 12-week time point, lamellar bone in the fracture/TBI group also had thicker appearance compared with the fracture-only group.

Immunohistochemical analysis

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

At 2 weeks after surgery, a greater percentage of leptin-positive cells was found in the fracture/TBI group compared with the fracture-only group, although this did not reach statistical significance (Table 1). At 4, 8 and 12 weeks, the

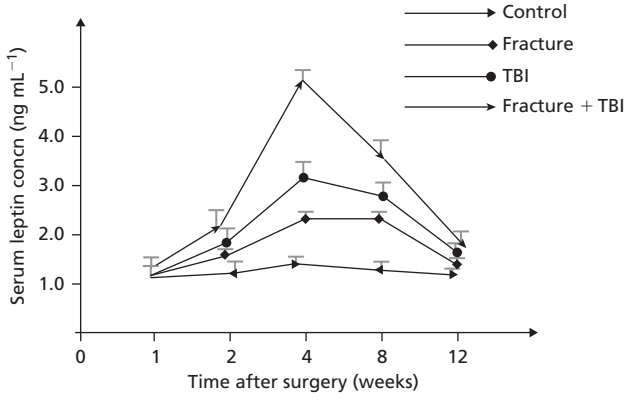

Figure 1 Leptin concentration in serum at weeks 1, 2, 4, 8 and 12 after surgery.

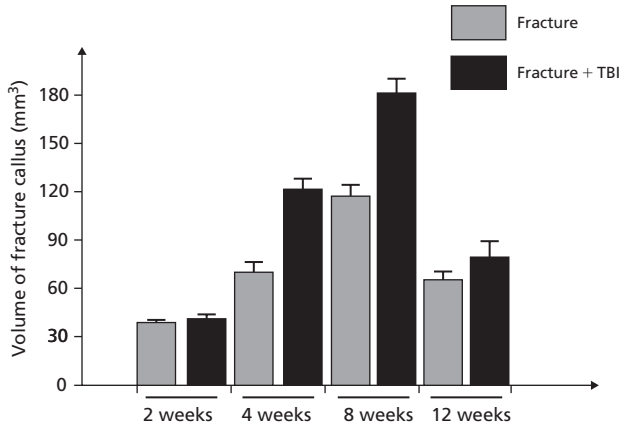

Figure 2 Volume of callus at weeks 2, 4, 8 and 12 in the fracture-only and fracture/TBI groups. At weeks 4 and 8 the volume of callus was significantly higher in the fracture/TBI group than in the fracture-only group ($P < 0.001$).

Table 1 Mean percentage of leptin-positive cells in the calluses of the fracture-only and fracture/TBI groups

Group	2 weeks	4 weeks	8 weeks	12 weeks
Fracture-only	8.5 ± 2.65	13.75 ± 2.50*	21.25 ± 2.88*	14.25 ± 2.98*
Fracture/TBI	11.75 ± 5.12	21.25 ± 4.35	33.75 ± 5.17	20.75 ± 2.06

Values are mean ± s.d. (n = 4).
*$P < 0.05$ fracture-only group vs fracture/TBI group at 4, 8 and 12 weeks.

Table 2 Number of leptin-positive cells per unit callus volume. Note the statistically significant increase in the fracture/TBI group at 2 and 12 weeks

Group	2 weeks	4 weeks	8 weeks	12 weeks
Fracture-only	0.23 ± 0.02*	0.17 ± 0.01	0.18 ± 0.03	0.21 ± 0.04*
Fracture/TBI	0.29 ± 0.03	0.19 ± 0.04	0.19 ± 0.02	0.26 ± 0.02

Values are mean ± s.d. (n = 4).
*$P < 0.05$ vs fracture/TBI group.

percentage of positive cells in the callus was significantly higher in the fracture/TBI group than in the fracture-only group ($P < 0.05$) (Table 1). This difference peaked at the 8-week time point and decreased at 12 weeks.

When analysing leptin-positive cells normalised to callus volume, the fracture/TBI group demonstrated a higher result at all time points, but the difference was significant only at 2 weeks and 12 weeks (Table 2).

Discussion

Leptin, the circulating protein product of the *Ob* gene, was initially discovered as a satiety factor regulating food intake and energy expenditure (Tartaglia 1997). It is produced primarily by adipocytes and acts on the hypothalamus; however, important non-adipocytic extra-hypothalamic pathways also exist (Fei et al 1997; Barr et al 1999). More recently, the role of leptin in bone metabolism has been identified (Takeda et al 2002; Thomas 2004) and is undergoing intensive characterisation, especially in the context of trauma and osteoporosis.

Leptin acts on the hypothalamus to increase sympathetic output, and this exerts an anti-osteogenic effect via β2-adrenoceptors on the surface of osteoblasts (Takeda et al 2002). Peripherally acting leptin, however, has an opposite effect by promoting bone mineralisation (Reseland et al 2001) and osteoblast-to-osteocyte differentiation (Thomas et al 1999; Gordeladze et al 2002). Furthermore, 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 (Ealey et al 2006). In the physiological resting state, however, the two opposing actions of leptin are in balance (Thomas & Burguera 2002).

The overall control of leptin action is not well understood, especially in the setting of fracture and TBI, where there is significant metabolic disturbance and inflammatory reaction. Linked in with this is the stress response of the neuroendocrine system where several cytokines and hormonal factors such as insulin and glucocorticoids have been shown to alter serum leptin levels (Kolaczynski et al 1996; Mantzoros et al 1996; Miell et al 1996; Utriainen et al 1996; Mantzoros et al 1997). TBI increases levels of pro-inflammatory cytokines and chemokines and activates a variety of endogenous inflammatory cells, including microglia and astrocytes (Smith 1994; Shahbazian et al 1999; Stamatovic et al 2006). These parenchymal cells contribute to an increase in endogenous pro-inflammatory modulators that can promote cellular responses to trauma and contribute to neuronal death. In particular, the pro-inflammatory cytokine IL-1 is induced rapidly after TBI (Fassbender et al 2000) and, notably, IL-1 is found to increase leptin levels (Janik et al 1997). This inflammatory pathway may underlie our findings of raised leptin in trauma.

Serum leptin was increased significantly in the TBI group, fracture group and fracture/TBI group compared with the control group from the second week after surgery ($P < 0.05$) (Figure 1). There are several possible explanations for the elevated leptin levels in the fracture-only condition. Firstly, the hypermetabolic response from injury generally results in mobilisation of more free fatty acids, which leads to leptin elevation by neuroendocrine feedback regulation (Faggioni et al 2000). Secondly, 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 (Lolmède et al 2003). Thirdly, affluent medulla ossium flava are released from the broken ends of fractured bone and because this bone marrow is composed of mainly adipose-degenerated haematopoietic cells, there is a local induction of leptin delivery (Lin et al 2006). Elevated serum leptin may then act peripherally to induce myeloid precursor cell differentiation and osteoblast proliferation, and accelerate the mineralisation of bone at the fracture site (Thomas et al 1999; Reseland et al 2001; Gordeladze et al 2002). As previously reported (Li et al 2001), 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 after injury. An interesting finding in the fracture/TBI group, which mimics the injuries and recovery response of the multi-trauma patient, was a significantly higher leptin level than in other treatment groups at 4 and 8 weeks (Figure 1). The number of leptin-positive cells and volume of callus in the fracture/TBI group were also significantly greater than in the fracture-only group. These results support the concept that injury to the hypothalamus may result in additional peripheral secretion of leptin and bone regeneration through loss of sympathetic inhibitory control (Rayner & Trayhurn 2001; Patel et al 2002; Wang et al 2005). After TBI, direct damage, including diffuse axonal injury, contusions, intracranial haematoma and pathophysiology, is amplified by secondary or delayed events including hypoxia, hypotension, ischaemia, oedema, and increased intracranial pressure. All of these secondary factors will lead to hypothalamic injury and dysfunction

(Wilcockson et al 2002). Singleton et al (2002) found that mild brain injury in mice resulted in early apoptotic neuronal death in the thalamus and cortex, indicating particular biomechanical vulnerability at these sites.

In addition to loss of sympathetic leptin inhibition, hypothalamic damage may impair the anti-osteogenic effects of leptin acting through this site (Takeda et al 2002), contributing to the enhanced bone formation observed in the fracture/TBI group.

TBI can increase sympathetic outflow in the acute period, but it is evident from our data that, with the passage of time, the peripheral effect of increasing leptin may outweigh any potential sympathetic inhibition of leptin and bone formation. The delay in a significant increase in serum leptin in the fracture/TBI group until week 4 after surgery may be related to the acute post-injury stress response and, specifically, early increases in sympathetic outflow. Dysautonomia and 'sympathetic storms' are well known to occur after TBI (Boeve et al 1998; Baguley et al 1999), and adrenergic stimulation acts to down-regulate leptin expression and secretion by adipocytes (Mantzoros et al 1996). Serum leptin may also be lower at an early stage because of fasting conditions in the injured patient or animal (Jeevanandam et al 1998). After a period of approximately 3 months, features of dysautonomia such as heart rate variability begin to resolve (Keren et al 2005) and at this point it is possible that sympathetic inhibition of both leptin and osteogenesis gives way to pro-osteogenic effects and the leptin increase noted with both TBI and specific lesions of the hypothalamus (Patel et al 2002; Trentz et al 2005). It is possible that an abatement of sympathetic instability occurred earlier in our animal model (2–4 weeks), given that the brain injury inflicted was a tightly regulated focused procedure rather than the often complex TBI sustained by human patients.

The peak in callus formation demonstrated at 8 weeks (Figure 2) with subsequent reduction corresponds with the known process of callus resorption, most likely by osteoclasts, facilitating consolidation and true bony healing (Schell et al 2006). The changes in callus volume also influence the normalised leptin-positive cell count (Table 2). In this case a significant difference was still noted between fracture-only and fracture/TBI groups but only at the 2- and 12-week time points. Lack of statistical difference at 4 and 8 weeks may be related to the increase in leptin-positive cells in the fracture/TBI group (Table 1) being offset by an accompanying increase in callus volume. Hence it may be misleading to consider the normalised data in isolation. Furthermore, we also found a positive correlation between leptin concentration in serum and volume of callus formation in the fracture/TBI group (Figure 3). From 2 to 8 weeks, the higher serum leptin level in the TBI/fracture group was accompanied by a larger callus volume, which may again suggest that leptin stimulates bone-forming cells.

To summarise our findings and conclude, leptin levels increase more significantly after combined TBI and fracture which may be related to hypothalamic damage and reduced inhibition of peripheral leptin secretion by the mechanisms discussed above. 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 4 weeks after injury, and this may relate to early inhibition by sympathetic activity seen in TBI. In future studies on leptin in TBI 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 TBI.

Our early findings should stimulate further investigation into the manipulation of peripheral leptin to either enhance fracture non-union, or potentially limit the complication of heterotopic bone formation seen in TBI patients.

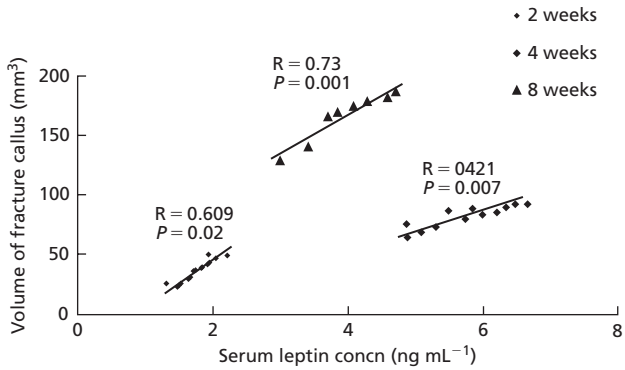

Figure 3 Positive correlation between leptin concentration in serum and volume of callus formation in the fracture/TBI group from 2 to 8 weeks.

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