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Case-Controlled, Cross-Sectional Evidence that Spinal Cord Injury Associated with Sympathetic Decentralisation may Alter Osteocalcin Signalling

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Abstract

Objective: Osteocalcin has been shown to exert an endocrine influence on bone and energy metabolism in both animals and humans; recent evidence indicating a regulatory role for osteocalcin in glucose homeostasis and energy metabolism and as a marker for bone metabolism.

The aim of this study was to investigate the interaction between osteocalcin, body composition, and blood biomarkers in men with spinal cord injury (SCI) compared with able-bodied male controls.

Materials and Methods: Twenty men with SCI were matched for age, height, and weight with 20 able-bodied controls. Body composition, bone density and blood levels of adiponectin, leptin, insulin, glucose, insulin-like growth factor-1 (IGF-1) were undertaken. Total body and regional fat mass (FM), fat free mass (FFM), total body bone mineral density (BMD) and circulating levels of blood biomarkers were compared between the two groups and correlation analyses performed between osteocalcin and all measures.

Results: Compared with controls, SCI had lower total and leg FFM ($P<0.05$), but higher total and regional FM ($P<0.05$). Osteocalcin negatively correlated with age ($P<0.05$), and positively with total, trunk and arm FFM, and IGF-1 ($P<0.05$) in SCI men. Negative correlations between osteocalcin and age ($P<0.05$) and positive correlations with total and all regional FM depots, leptin, fasting glucose, and IGF-1 ($P<0.05$) were found for the controls.

Conclusions: Crosstalk between fat mass, osteocalcin, glucose metabolism and adipokines is lost with decentralisation in the sympathetic nervous system (SNS). The clinical impact of this decentralisation deserves further investigation. These findings do not imply causality, but should be considered hypothesis generating.

Keywords: Body composition; Sympathetic nervous system; Adipocytokines; Glucose metabolism

Abbreviations:

BMD: Bone Mineral Density; BMI: Body Mass Index; CV: Coefficient of Variation; DXA: Dual Energy X-ray Absorptiometry; FFM: Fat Free Mass; FM: Fat Mass; GSK3β: Glycogen Synthase Kinase 3 Beta; IGF-1: Insulin-like Growth Factor-1; IRMA: Immunoradiometric Assay; RIA: Radioimmunoassay; SCI: Spinal Cord Injured; SNS: Sympathetic Nervous System; VMH: Ventromedial Hypothalamus

Introduction

The skeleton has many functions within the human body. Recent research brings this organ system, like that of adipose tissue, to the fore in key areas of metabolic regulation. Indeed, it now appears that harmonious crosstalk between bone and adipose tissue organ systems is required for metabolic homeostasis, while dysregulated crosstalk may be instrumental in disease development [1-6]. Perhaps unsurprisingly, muscle-secreted myokines have also been identified as active regulators of metabolic function [7-9].

Key components of this metabolic control are the bone-derived osteokines, osteocalcin, adipose tissue-derived adipokines, leptin and adiponectin, and the sympathetic nervous system (SNS) [1,10,11]. Osteocalcin, a secretory product derived from osteoblasts, has been shown to exert an unexpected, but important, endocrine influence on bone and energy metabolism in both animals and humans [2-5]. As a marker of bone metabolism, osteocalcin levels are used to ascertain bone formation activity. However, recent evidence suggests osteocalcin also plays a regulatory role in glucose homeostasis and energy metabolism [12]. Mice lacking osteocalcin demonstrate abnormally high adipose tissue levels, hyperglycaemia, and hypoinsulinaemia when compared with wild type littermates and this adverse metabolic phenotype was heavily influenced by a significant reduction in adiponectin, an adipokine known to affect insulin sensitivity [4]. The other important adipokine, leptin, has been identified as a mediator of osteocalcin release from osteoblasts, via the sympathetic nervous system (SNS) [1]. Once released, osteocalcin acts directly on the pancreas to increase insulin sensitivity and β-cell proliferation, and indirectly through adiponectin, to improve insulin

sensitivity in muscle, liver and fat [1]. Thus it appears bone and adipose tissue function synergistically in regulating many biological processes. Additionally, active crosstalk between adipokines and muscle-derived cytokines has been reported [9]; with this interaction implicated in the development of obesity [7]. Given that osteoblasts, adipocytes and myocytes originate from the same stem cell lineage, the finding of regulatory cross-talk between the secretory products of these cells is not surprising.

While the association between bone and glucose metabolism has been most frequently investigated in cell and animal models, human studies of the bone/adipose tissue control of energy metabolism reveal similar patterns. Findings in humans that osteocalcin levels are related to insulin sensitivity, body mass index (BMI), fat mass, age, fasting glucose concentration, muscle glucose utilisation, leptin and adiponectin, indicate that a link between bone and energy metabolism is not confined to animal models [5,6,13-15]. Furthermore, low osteocalcin levels have been observed in obese, elderly and individuals with Type 2 diabetes and correlates with an adverse metabolic phenotype [3,5,15-19].

Injury to the spinal cord results in sympathetic decentralisation, meaning a loss of sensory and motor functions via the central and peripheral afferent and efferent pathways [20,21]. Disruption can cause pathologic changes in the SNS, resulting in altered responses to central and peripheral SNS stimulation [20,22]. Alterations in sympathetic tone can have adverse effects on bone, glucose and energy metabolism [23,24]. Leptin stimulates sympathetic tone, which decreases osteocalcin bioactivity and subsequently inhibits insulin secretion, causing bone loss, and alteration in energy metabolism [23,24]. As the SNS is a key component of the signalling between bone and other tissues, we investigated the interaction between osteocalcin and body composition, adipokines, blood markers of glucose control, and IGF-1 in men with spinal cord injury compared with age-, height-, and weight-matched able-bodied male controls.

Methods

Subjects

Twenty men (aged between 16 and 52 years) who had sustained a traumatic injury to the spinal cord participated in this study, which had ethical approval from the Ethics Committees of the Health Funding Authority, Otago and Canterbury. Written, informed consent was obtained from all participants. All SCI males had sustained their injury for longer than one year. Within the SCI group, there were 13 tetraplegic participants (lesion levels, C4–C7). The neurological status of five tetraplegic men was classified as American Spinal Injury Association (ASIA) A; (Frankel A); six ASIA B; One ASIA C and one ASIA D. Seven paraplegic participants had sustained a spinal cord lesion between T5-L3; four ASIA A; one ASIA B and two ASIA C. Seven paraplegic participants had sustained a spinal cord injury lesion between T5–L5. Four were complete, Frankel A; one incomplete Frankel B; and two incomplete, Frankel C. Their mean duration of injury was 10.3 ± 1.8 years. Each SCI participant was age-, height-, and weight-matched with an able-bodied control male. Control participants were matched within five years of age, 5cm of height and, where possible, within 5 kg of weight of the spinal injured participants. None of the SCI or able-bodied participants was diabetic or reported using medications known to affect carbohydrate or lipid metabolism.

Data Collection

Total body and regional fat free mass (FFM), fat mass (FM) and total bone mineral density (BMD) were measured in all participants using DXA (LUNAR DPX-L, Lunar Corporation, Madison, WI). Our *in vivo* coefficients of variation for DXA scanning precision for 10 consecutive scans were 2.6% for total FM, 2.5% for total body mass expressed as a percentage of total body mass, and <3.5% for all regional measures of FM. Body weight in both groups was taken as the sum of total fat tissue; total fat free tissue; and total bone mineral content from the total body DXA scan. Physical activity was ascertained using an in-house lifestyle questionnaire that asked all individuals to itemise the average minutes per week spent undertaking voluntary physical activity.

Plasma samples were collected and stored at -80°C for analysis. Stored samples were analysed for osteocalcin, leptin, adiponectin, insulin, glucose, and insulin-like growth factor-1 (IGF-1). Osteocalcin, leptin, adiponectin, and IGF-1 analyses were performed by Endolab (Canterbury Health Laboratories, Christchurch, New Zealand). Glucose and insulin samples were analysed in the Department of Human Nutrition Lipid and Mineral Testing Laboratory at the University of Otago. Osteocalcin was analysed using an immunoradiometric assay (IRMA) automated method (Roche Elecsys 2010, Indianapolis, USA). The co-efficient of variation (CV) for this assay is 13%. Leptin results were obtained using a human leptin radioimmunoassay (RIA) kit (Linco Research Inc., St Charles, MO, USA). Assay CV's were 7.5% at 61.6 μg/l; 4% at 15.9 μg/l; and 9.7% at 2.3 μg/l. Adiponectin was measured using a radioimmunoassay method (Linco Research, St Charles, MO, USA). Intra-assay CV was 8.8%. An "in house" RIA was used to determine IGF-I levels (Canterbury Health Laboratories, Christchurch, New Zealand). Briefly, this method involves using an acid-ethanol extraction procedure followed by cryoprecipitation (-20°C) to ensure minimal interference in the assay from insulin-like growth factor binding proteins [25]. Once the supernatant is decanted and diluted, IGF-II antiserum is added to bind any remaining binding proteins still present following the extraction procedure (Canterbury Health Laboratories, Christchurch, New Zealand). Standards are prepared from receptor grade IGF-I (Gropep Ltd, Adelaide, Australia). Following overnight incubation (4°C) ^{125}I-IGF-I (Gropep Ltd) is added and a further period of incubation precedes the separation step. Bound and free IGF-I are separated after adding a second antibody (γ-globulin) and polyethylene glycol. The supernatant is then aspirated and the radioactivity of the pellet counted. Inter-assay coefficient of variation (CV) is 12-15%, and intra-assay CV is 5.5-13%.

Glucose was analysed using the hexokinase method. Analysis kits were obtained from Roche (Unimate 5, Roche Diagnostics Corporation, Indianapolis, USA) and analysed on a Cobas Mira Plus auto-analyser (Roche Diagnostics Corporation, Indianapolis, USA). Coefficient of variation (CV) for this assay was 3.5%. Insulin was analysed by radioimmunoassay (RIA), using the Coat-A-Count assay (Diagnostic Products Corporation, Los Angeles, CA), on a Cobas Mira Plus auto-analyser (Roche Diagnostics Corporation, Indianapolis, USA); CV for this assay was 7%.

Statistical Analysis

Student t-tests were used for comparisons between SCI and controls for descriptive statistics and all blood and body composition measures. Pearson product moment correlation coefficients were used to

determine associations between osteocalcin, blood measures, and body composition in each group. Partial correlation coefficients controlling for age were undertaken between osteocalcin and blood and body composition measures for both groups to determine the influence of age on the significant correlations identified in the correlation analysis. Statistical analyses were undertaken using Statistical Package for the Social Sciences (SPSS v16.0, Chicago, Illinois, USA). Statistical significance was accepted at $p<0.05$.

Results

Demographic details for all participants are presented in Table 1. There were no significant differences ($p>0.05$) between the groups for age, height, weight, or BMI. Similarly, physical activity levels were not significantly different between the groups. Four SCI and four controls reported physical activity of <60 minutes/week, while the remainder of men in both groups were highly physically active, many being National or International Sporting representatives. DXA scans revealed significant differences ($p<0.05$) between the groups for total FM, total FFM, body fat expressed as a percentage of total body mass, and total BMD, all measures of regional FM and FFM and leptin (Table 2); despite similar levels of testosterone in both groups(data not shown). Plasma levels of osteocalcin, adiponectin, fasting glucose, fasting insulin, and IGF-1 were not significantly different ($p>0.05$) between the SCI and control groups (Table 2). Leptin and fasting insulin values violated normal distribution and were log transformed for all analyses. Raw data are presented in the tables for ease of understanding.

Pearson correlation coefficients revealed significant associations ($p<0.05$) between osteocalcin and age, total FFM, trunk and arm FFM and IGF-1 in the SCI group, while significant correlations ($p<0.05$) were observed for osteocalcin and age, total and all regional measures of FM and body fat percentage, leptin, fasting plasma glucose and IGF-1 in the control group (Table 3). After adjusting for age, significant associations ($p<0.05$) between osteocalcin and total FFM, trunk FFM, and arm FFM remained in the SCI group, while only IGF-1 remained significantly associated ($p<0.05$) with osteocalcin levels in the controls (Table 4).

Variable	SCI	Control	Significance (p value)
Age (years)	33 ± 2	33 ± 2	0.9
Weight (kg)	75.8 ± 3.1	77.2 ± 1.6	0.7
Height (m)	1.80 ± 0.01	1.79 ± 0.01	0.6
BMI (kg/m^2)	23.5 ± 1.0	24.1 ± 0.4	0.6
Physical activity (mins/wk)	376 ± 59	312 ± 46	0.4

Table 1: Demographic details for spinal cord injured (SCI, n=20) and control (n=20) participants. Data are mean ± SEM.

Variable	SCI	Control
Total FFM (kg)	51.4 ± 1.9	60.0 ± 1.0†
Trunk FFM (kg)	25.9 ± 0.9	27.0 ± 0.5
Leg FFM (kg)	14.6 ± 0.8	21.6 ± 0.5*
Arm FFM (kg)	7.2 ± 0.4	7.6 ± 0.2
Total FM (kg)	21.6 ± 2.4	13.9 ± 1.3†
Trunk FM (kg)	11.2 ± 1.3	7.1 ± 0.8*
Leg FM (kg)	7.0 ± 0.8	4.9 ± 0.4*
Arm fat (kg)	2.2 ± 0.3	1.3 ± 0.2†
Body fat (%)	27.5 ± 2.3	17.8 ± 1.5†
Total BMD (g/cm^2)	1.12 ± 0.02	1.27 ± 0.02†
Osteocalcin (µg/l)	27.6 ± 2.6	31.4 ± 2.8
Leptin (µg/l)	6.1 ± 1.1	2.7 ± 0.4†
Adiponectin (mg/l)	11.9 ± 1.2	9.6 ± 1.0
Fasting glucose (mg/dl)	93.7 ± 1.8	99.1 ± 1.8
Fasting insulin (µIU/ml)	8.1 ± 1.0	7.2 ± 0.7
IGF-1 (µg/l)	180.0 ± 16.6	182.2 ± 9.8

Table 2: Body composition and blood values for spinal cord injured (SCI) and control participants. Data are mean ± SEM. FFM=lean tissue mass; FM=fat mass; BMD=bone mineral density; IGF-1=insulin-like growth factor-1. *significant difference between groups at $p<0.05$; †significant difference between groups at $p<0.01$. Conversion factors: osteocalcin, µg/l*0.171=nmol/l; leptin, µg/l*0.0625=nmol/l; glucose, mg/dl*0.0555=mmol/l; insulin, µIU/ml*6.945=pmol/l; IGF-1, µg/l*0.13=nmol/l; conversion factor not available for adiponectin.

Discussion

To the best of our knowledge, this is the first paper to investigate the relationship of osteocalcin with body composition, adipokines and markers of carbohydrate metabolism in men with spinal cord injury; all of whom were closely matched with able-bodied controls. Results of correlation analyses between osteocalcin and body composition and blood variables in our control group were similar to those reported elsewhere;[5,15,17-19] however, results in the SCI group suggest that disruption to the central nervous system alters the manner in which adipose tissue, muscle and bone may communicate.

Spinal cord injury results in decentralisation of the SNS, the extent of which is dependent upon the level of the lesion, and the degree to which sensory and motor functions are affected [20,26]. Loss of SNS activity in non-SCI individuals has been implicated in fat gain and altered patterns of energy metabolism [1,27,28]. Changes in body tissue compartments in the SCI group occurred despite a high level of physical activity. The SNS has been identified as a key regulator of energy metabolism, fat mass, and insulin sensitivity via osteocalcin, an osteoblast-specific protein and two adipose tissue-derived adipokines, leptin and adiponectin [1,4,5,13,15,17,19].

Osteocalcin and adiponectin levels were similar between our SCI and control groups; however, leptin concentrations were higher in the SCI group. Higher leptin levels in SCI have been reported by others [29-32]. As leptin is secreted by adipocytes, it is of little surprise that as fat mass increases, leptin levels will also rise; our SCI group had higher measures of total and regional fat mass than their able-bodied controls. Leptin was significantly associated with total and regional fat

mass in both SCI and control groups (data not shown). However, Jeon et al. reported that after adjusting for fat mass, leptin levels were 45% higher in SCI compared to able-bodied controls; suggesting that factors other than fat mass contribute to leptin secretion in SCI individuals [33]. The most obvious factor is disruption to the central nervous system as a result of spinal cord injury.

Variable	SCI	Control
Age	-.59†	-.64†
Total FFM (kg)	.48*	.28
Trunk FFM (kg)	.54*	-.17
Leg FFM (kg)	.30	0.42
Arm FFM (kg)	.49*	-.01
Total FM (kg)	-.11	-.59†
Trunk FM (kg)	-.13	-.58†
Leg FM (kg)	-.14	-.56*
Arm fat (kg)	.12	-.49*
Percent total FM	-.31	-.62†
Total BMD (g/cm2)	0.14	-.25
Leptin (µg/l)	-.36	-.54*
Adiponectin (mg/l)	.01	.16
Fasting glucose (mg/dl)	-.14	-.49*
Fasting insulin (µIU/ml)	.33	-.06
IGF-1 (µg/l)	.61†	.55*

Table 3: Pearson product moment correlation coefficients between osteocalcin and body composition and blood measures for spinal cord injured (SCI) and control participants.FFM=lean tissue mass; FM=fat mass; IGF-1=insulin-like growth factor-1. *correlation is significant at $p<0.05$; or †$p<0.01$ (2-tailed).

Variable	SCI	Controls
IGF-1		.40*
Total FFM	.39*	
Trunk FFM	.47*	
Arm FFM	.42*	

Table 4: Partial correlation coefficients between osteocalcin and body composition and blood measures, controlling for age in spinal cord injured (SCI) and control participants. FFM=lean tissue mass; IGF-1=insulin-like growth factor-1. *correlation is significant at $p<0.05$ (1-tailed).

While osteocalcin has been identified as a skeletal mediator between leptin and SNS activity, regulating fat mass, energy metabolism and insulin sensitivity [1], no association between osteocalcin and any measure of fat mass, leptin, glucose, or insulin was observed in the SCI group. For leptin to exert its effects, an intact SNS is required [33], thus our findings that osteocalcin did not correlate with fat or metabolic measures in the SCI participants lends support to this hypothesis, as leptin communicates with osteocalcin via the SNS to regulate insulin sensitivity, glucose uptake and energy metabolism [1]. However, as reported elsewhere, an inverse association between osteocalcin and all measures of fat mass, leptin, and fasting plasma glucose was observed in the control group [5,13,15,17,19]. Also in agreement with others, statistical significance for this group was lost following age-adjustment for these variables [19], although the positive association with IGF-1 remained following age-adjustment; indicating the independent effects of this potent growth factor on the skeleton [34].

Novel findings in our study are the positive associations between osteocalcin and total, trunk and arm fat free mass and IGF-1 in the SCI group. Other than IGF-1, these associations remained significant following age-adjustment. No significant associations between osteocalcin and FFM have been reported in any population studied to date. While the SNS is implicated in osteocalcin's effects on energy metabolism and insulin sensitivity, it appears osteocalcin may also exert its effects peripherally. Support for this hypothesis was demonstrated in four-week-old mice, where gold thioglucose was used to destroy neurons of the ventromedial hypothalamus (VMH); an area of the brain important in bodyweight and glucose regulation [2]. Destruction of the VMH led to glucose intolerance, increased fat pad weight and insulin resistance [2]. Infusion of osteocalcin re-established glucose tolerance, insulin sensitivity and bodyweight regulation in the VMH-lesioned mice, which suggests osteocalcin can act through receptors present on target cells [2]. Muscle is one of the target tissues influenced by osteocalcin, which increases insulin-sensitivity and glucose uptake in this tissue [12]. More recently, Brotto et al. [35], investigating osteoporosis and sarcopenia, found that osteokines secreted by bone are detected by muscle and that in turn, muscle-derived myokines are detected by bone, affecting bone mass and strength. This group also report that different muscle types, cardiac, skeletal and smooth muscle, secret different signals for different reasons [35]. Alterations in the Wnt signalling pathway, which is important in normal development and muscle regeneration [36], has been suggested as a possible link with wasting diseases in muscle and bone [35]. Wnt signalling may be activated through IGF-1 [37], which leads to the inhibition of glycogen synthase kinase 3 beta (GSK3β), a downstream mediator [38]. Inhibition of GSK3β induces protein synthesis and blocks pathways associated with muscle atrophy; GSK3β inhibition is also a pivotal determinant for myogenesis [36]. The positive association between osteocalcin and FFM observed in the SCI group suggests that crosstalk between bone and muscle may occur though IGF-1 and/or pathways associated with IGF-1 signalling to preserve FFM in the presence of SNS decentralisation. This hypothesis may have some merit given that IGF-1-stimulated secretion of the carboxylated form of osteocalcin is closely associated with bone formation and mineralization [39]. We measured total osteocalcin levels, thus future research needs to identify the independent and/or reciprocal effects of carboxylated and undercarboxylated forms of osteocalcin on muscle protein synthesis. A lower ratio of undercarboxylated to carboxylated osteocalcin negatively correlated with BMI in obese individuals suggesting a pathophysiological link with an imbalance between these two forms of osteocalcin [3].

Limitations in our study include the small sample size and the combination of individuals with differing levels of neurological deficit that comprised the SCI group. Lesion levels above T6 are associated with increased leptin levels and decreased SNS activity [32]; however,

removal of the four SCI participants with lesions below T6 did not significantly alter the original findings (data not shown). We are confident that the heterogeneity of the SCI group, with respect to lesion level and degree of completeness, did not impact on the results observed for body composition as the extent of neurological deficit has not been found to impact significantly on these measures within SCI groups [40]. A further limitation is that we did not measure levels of undercarboxylated osteocalcin; this form is more consistently associated with the protective metabolic effects of osteocalcin [41]. However, the carboxylated form of osteocalcin has been associated with insulin resistance, decreased glucose transport, metabolic syndrome and measures of fat mass in humans [5,6,15,19]. Additionally, total adiponectin, rather than the high-molecular weight forms of adiponectin was measured in our study; the latter forms are suggested to be a more sensitive measure of metabolic regulation [42].

In conclusion, we found that a similar plasma concentration of osteocalcin was differentially associated with metabolic markers and body composition in men with spinal cord injury compared with their age-, height-, and weight-matched able-bodied controls. It appears decentralisation in the SNS may alter the way in which bone and fat, bone and muscle, and fat and muscle may communicate to maintain metabolic homeostasis. Our results are not intended to imply causality, but should be considered hypothesis generating. Further investigation is required to elucidate the impact of sympathetic decentralisation on the interaction of osteokines, adipokines and myokines and their influence metabolic control and body tissue regulation.

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