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Research Paper

Monocyte chemotactic protein-1 deficiency attenuates and high-fat diet exacerbates bone loss in mice with Lewis lung carcinoma

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ABSTRACT

Bone loss occurs in obesity and cancer-associated complications including wasting. This study determined whether a high-fat diet and a deficiency in monocyte chemotactic protein-1 (MCP-1) altered bone structural defects in male C57BL/6 mice with Lewis lung carcinoma (LLC) metastases in lungs. Compared to non-tumor-bearing mice, LLC reduced bone volume fraction, connectivity density, trabecular number, trabecular thickness and bone mineral density and increased trabecular separation in femurs. Similar changes occurred in vertebrae. The high-fat diet compared to the AIN93G diet exacerbated LLC-induced detrimental structural changes; the exacerbation was greater in femurs than in vertebrae. Mice deficient in MCP-1 compared to wild-type mice exhibited increases in bone volume fraction, connectivity density, trabecular number and decreases in trabecular separation in both femurs and vertebrae, and increases in trabecular thickness and bone mineral density and a decrease in structure model index in vertebrae. Lewis lung carcinoma significantly decreased osteocalcin but increased tartrate-resistant acid phosphatase 5b (TRAP 5b) in plasma. In LLC-bearing mice, the high-fat diet increased and MCP-1 deficiency decreased plasma TRAP 5b; neither the high-fat diet nor MCP-1 deficiency resulted in significant changes in plasma concentration of osteocalcin. In conclusion, pulmonary metastasis of LLC is accompanied by detrimental bone structural changes; MCP-1 deficiency attenuates and high-fat diet exacerbates the metastasis-associated bone wasting.

INTRODUCTION

Metastasis is the most devastating aspect of cancer, which is accompanied by wasting that eventually results in cachexia characterized by a significant skeletal muscle loss and multi-organ functional failures. Limited studies indicate that bone loss can occur during cancer-associated wasting. For example, lung cancer patients with a 30% loss of body mass exhibit significantly lower total body mineral content than healthy-matched controls [1]. Concurrent muscular and bone deterioration is found in a murine model of cancer cachexia [2]. Furthermore, pathways that induce muscular wasting may promote bone loss during cachexia [3].

Monocyte chemotactic protein-1 (MCP-1) is primarily considered a potent chemotactic factor that attracts monocytes and other inflammatory cells to the site of inflammation during tissue injury and infection [4]. However, MCP-1 has functions beyond tissue repair; it participates in the development and progression of many pathophysiological conditions, including cancer [5–7]. Elevated expression of MCP-1 is associated with poor outcomes and short disease-free intervals [5–7], and thus it has prognostic value for cancer patients. In support of the clinical observations, animal studies show that MCP-1 participates in primary tumor growth and metastasis [8–10]. In addition, MCP-1 is pro-osteoclastogenic. High expression of MCP-1 is found in osteoporotic bone of humans [11]. *In vitro* studies show that MCP-1 stimulates the formation of osteoclasts [12].

Adipose tissue produces proinflammatory cytokines including MCP-1. Adipose tissue expression of MCP-

Table 1: Trabecular structural changes in femurs of MCP-1$^{-/-}$ and wild-type mice fed the AIN93G or the high-fat diet

	AIN93G Wild-type No LLC	AIN93G Wild-type	AIN93G MCP-1$^{-/-}$	High-fat Wild-type	High-fat MCP-1$^{-/-}$	Diet	*p* values Gene	D × G
BV/TV, %	14.8 ± 1.0 *	10.5 ± 0.8	12.7 ± 0.9	9.8 ± 0.6	10.8 ± 0.5	0.10	< 0.05	0.41
Conn.D, 1/mm^3	156.4 ± 12.9 *	108.3 ± 12.9	144.2 ± 12.0	90.3 ± 7.5	109.4 ± 8.4	< 0.05	< 0.05	0.44
SMI	2.2 ± 0.1	2.6 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	0.22	0.12	0.37
Tb.N, 1/mm	5.4 ± 0.1 *	4.8 ± 0.1	5.3 ± 0.1	4.5 ± 0.1	4.8 ± 0.1	< 0.01	< 0.01	0.30
Tb.Th, µm	46.5 ± 1.0 *	41.9 ± 0.6	42.6 ± 1.1	43.5 ± 0.8	43.3 ± 0.7	0.19	0.76	0.63
Tb.Sp, µm	181.5 ± 4.1 *	207.0 ± 6.3	186.0 ± 4.0	219.4 ± 5.3	206.9 ± 3.6	< 0.01	< 0.01	0.38

Two-way ANOVA was performed to compare differences among the groups of LLC-bearing mice. *A priori* contrasts were performed to compare the difference between AIN93G-fed wild-type mice with or without Lewis lung carcinoma (LLC); *p<0.01 compared to AIN93G wild-type. Values are means ± SEM (n = 14-15 per group). BV/TV: bone volume fraction; Conn.D: connectivity density; SMI: structure model index; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation.

1 mRNA correlates positively with adiposity and body mass index [13]. Accumulation of visceral fat mass is an indicator of detrimental health outcomes in obesity [14, 15]. Being obese at the time of primary cancer diagnosis is predictive of poor prognosis [16–19]. Increased body weight caused by obesity was considered a benefit to bone health because of reported positive correlations between body weight and bone mineral density [20]. However, studies also show an inverse relationship between body fat mass and bone mass in humans [21]. Laboratory studies show that consumption of a high-fat diet results in bone loss in rodent models [22–24].

In our study of the relationship between obesity and metastasis, we found that consumption of a high-fat diet enhances and MCP-1 deficiency reduces spontaneous pulmonary metastasis of Lewis lung carcinoma (LLC) in mice [25]. Furthermore, we found bone loss in mice with lung metastases of LLC [24]. Thus, we hypothesized that a high-fat diet exacerbates and MCP-1 deficiency alleviates metastasis-associated bone wasting. To test this hypothesis, we performed micro-computed tomographic analysis of femurs and vertebrae collected from LLC-bearing wild-type and MCP-1$^{-/-}$ mice in a previous study [25] showing that the high-fat diet enhances and MCP-1 deficiency attenuates metastasis of LLC.

RESULTS

Physical measurement of bone

There were no differences in femur length, medial-lateral axis width and anterior-posterior axis width between non-tumor-bearing and LLC-bearing wild-type mice fed the AIN93G diet (data not shown). In LLC-bearing mice, the femur length of MCP-1$^{-/-}$ mice was slightly but significantly shorter than that of wild-type mice (14.53 ± 0.08 vs. 14.82 ± 0.08 mm, p<0.05); there were no differences in medial-lateral axis width and anterior-posterior axis width between MCP-1$^{-/-}$ and wild-type mice (data not shown). The high-fat diet did not change these variables compared to the AIN93G diet (data not shown).

Micro-computed tomographic measurement of trabecular bone

The presence of LLC was detrimental to the three-dimensional microstructure of trabecular bone. Compared to non-tumor-bearing mice, LLC reduced bone volume fraction (BV/TV) by 29%, connectivity density (Conn.D) by 31%, trabecular number (Tb.N) by 11%, and trabecular thickness (Tb.Th) by 10% and increased trabecular separation (Tb.Sp) by 14% in femurs (Table 1). In lumbar vertebra 4, LLC reduced BV/TV by 19% and Tb.Th by 10% and increased structure model index (SMI) by 30% and Tb.Sp by 6% (Table 2). Furthermore, LLC reduced bone mineral density (BMD) by 17% in femurs (Figure 1a) and 12% in vertebrae (Figure 1b). Pearson correlation analysis showed that the volume of lung metastases correlated inversely with BV/TV, Conn.D, Tb.N and BMD and positively with SMI and Tb.Sp in both femurs and vertebrae (Table 3).

The high-fat diet exacerbated the detrimental structural changes in trabecular bone mediated by LLC. Consuming the high-fat diet instead of the AIN93G diet decreased Conn.D by 21%, Tb.N by 8% and increased Tb.Sp by 9% in femurs (Table 1). The high-fat diet instead of the AIN93G diet increased SMI by 12% in vertebrae (Table 2). The high-fat diet decreased BMD by 12% in femurs (Figure 1a) but only marginally reduced BMD in vertebrae (Figure 1b).

Table 2: Trabecular structural changes in vertebrae of MCP-1$^{-/-}$ and wild-type mice fed the AIN93G or the high-fat diet

	AIN93G Wild-type No LLC	AIN93G Wild-type	AIN93G MCP-1$^{-/-}$	High-fat Wild-type	High-fat MCP-1$^{-/-}$	*p* values Diet	*p* values Gene	*p* values D × G
BV/TV, %	23.7 ± 0.9 *	19.3 ± 1.3	22.1 ± 0.8	18.4 ± 0.5	20.8 ± 0.7	0.21	< 0.01	0.79
Conn.D, 1/mm^3	267.2 ± 5.8	271.0 ± 8.1	297.1 ± 7.8	260.9 ± 7.0	283.1 ± 9.0	0.12	< 0.01	0.80
SMI	1.0 ± 0.1 *	1.3 ± 0.1	1.2 ± 0.10	1.5 ± 0.1	1.3 ± 0.1	0.05	< 0.05	0.54
Tb.N, 1/mm	5.7 ± 0.1	5.6 ± 0.1	5.7 ± 0.1	5.5 ± 0.1	5.6 ± 0.1	0.19	< 0.05	0.68
Tb.Th, µm	47.5 ± 1.0 *	42.8 ± 0.9	44.8 ± 0.7	42.7 ± 0.6	44.1 ± 0.9	0.65	< 0.05	0.69
Tb.Sp, µm	166.6 ± 1.7 *	175.7 ± 2.5	166.0 ± 2.1	177.2 ± 2.1	172.7 ± 2.6	0.07	< 0.01	0.25

Two-way ANOVA was performed to compare differences among the groups of LLC-bearing mice. *A priori* contrasts were performed to compare the difference between AIN93G-fed wild-type mice with or without Lewis lung carcinoma (LLC); *p<0.01 compared to AIN93G wild-type. Values are means ± SEM (n = 14-15 per group). BV/TV: bone volume fraction; Conn.D: connectivity density; SMI: structure model index; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation.

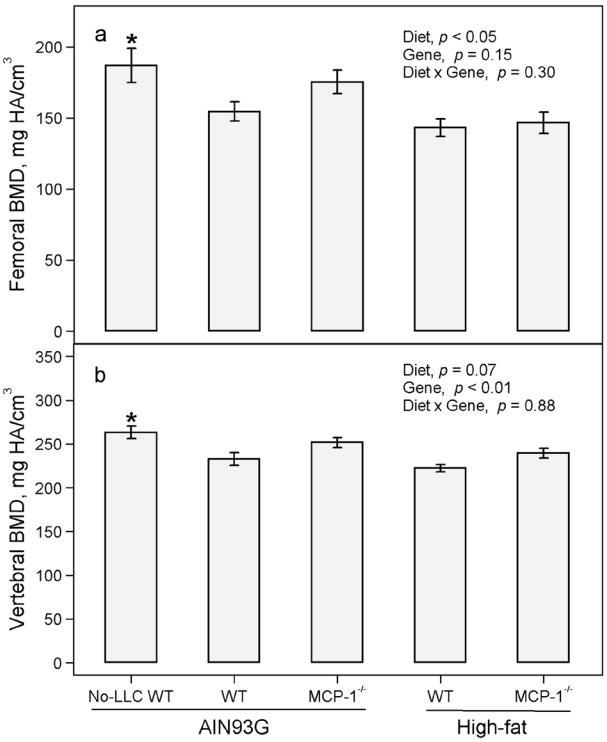

Figure 1: Bone mineral density of femurs and vertebrae of MCP-1$^{-/-}$ and wild-type mice fed the AIN93G or the high-fat diet. Two-way ANOVA were performed to compare differences among the groups of LLC-bearing mice. *A priori* contrasts were performed to compare differences between AIN93G-fed wild-type (WT) mice with or without Lewis lung carcinoma (No-LLC); *p<0.05 compared to AIN93G WT. Values are means ± SEM (n = 14-15 per group). BMD: bone mineral density.

Deficiency in MCP-1 attenuated detrimental structural changes in trabecular bone induced by LLC. The MCP-1 deficiency increased BV/TV by 16%, Conn.D by 28%, Tb.N by 9% and decreased Tb.Sp by 8% in femurs (Table 1). In vertebrae, MCP-1 deficiency increased BV/TV by 14%, Conn.D by 9%, Tb.N by 2% and Tb.Th by 4% and decreased SMI by 11% and Tb.Sp by 4% (Table 2). Deficiency in MCP-1 increased BMD in vertebrae by 8% (Figure 1b) but not in femurs (Figure 1a).

Micro-computed tomographic measurement of cortical bone

Compared to non-tumor-bearing mice, LLC-bearing mice exhibited decreases of 4% and 6% in cortical area fraction (Ct.Ar/Tt.Ar) and 7% and 9% in cortical thickness (Ct.Th) in femur mid-shaft and vertebrae, respectively (Table 4). The high-fat diet did not change LLC-induced decreases in Ct.Ar/Tt.Ar and Ct.Th in either femurs or vertebrae (Table 4). Deficiency in MCP-1 resulted in a slight but significant increase in Ct.Ar/Tt.Ar in femurs but not in vertebrae, nor did it increase Ct.Th in either femurs or vertebrae (Table 4).

Concentrations of osteocalcin and tartrate-resistant acid phosphatase 5b (TRAP 5b) in plasma

Compared to non-tumor bearing mice, LLC resulted in a 27% decrease in osteocalcin (Figure 2a) and a 50% increase in TRAP 5b in plasma (Figure 2b). There was no difference in osteocalcin between the high-fat and AIN93G diets and between MCP-1$^{-/-}$ and wild-type mice

Table 3: Pearson correlations of the extent of lung metastasis with trabecular structural changes in femurs and vertebrae of MCP-1$^{-/-}$ and wild-type mice fed the AIN93G or the high-fat diet

	Femurs				Vertebrae			
	Number of metastasis		Volume of metastasis		Number of metastasis		Volume of metastasis	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
BV/TV, %	–0.18	0.20	–0.48	< 0.01	–0.14	0.32	–0.40	< 0.01
Conn.D, 1/mm^3	–0.21	0.13	–0.54	< 0.01	–0.20	0.17	–0.32	< 0.05
SMI	0.09	0.52	0.43	< 0.01	0.25	0.07	0.41	< 0.01
Tb.N, 1/mm	–0.30	< 0.05	–0.60	< 0.01	–0.14	0.34	–0.33	< 0.05
Tb.Th, µm	0.02	0.92	–0.07	0.60	–0.13	0.38	–0.34	< 0.05
Tb.Sp, µm	0.28	< 0.05	0.60	< 0.01	0.13	0.38	0.33	< 0.05
BMD, mg HA/cm^3	–0.23	0.11	–0.51	< 0.01	–0.23	0.11	–0.46	< 0.01

Abbreviations: BV/TV: bone volume fraction; Conn.D: connectivity density; SMI: structure model index; Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; BMD: bone mineral density.

(Figure 2a). The high-fat diet compared to the AIN93G diet increased TRAP 5b by 26% (Figure 2b); MCP-1 deficiency decreased TRAP 5b by 20% compared to wild-type mice (Figure 2b).

DISCUSSION

The present study showed that the presence of LLC and its pulmonary metastases deteriorated trabecular and cortical structure and reduced bone mineral density in mice, indicating the significance of malignancy in bone wasting. The high-fat diet, which leads to increases in adipose MCP-1 [25], exacerbated and deficiency in MCP-1 attenuated LLC-mediated bone deterioration. These findings indicate that MCP-1 contributes to cancer-associated bone loss.

Lewis lung carcinoma metastasizes to lungs from a subcutaneous primary tumor [25, 26]. The present study showed that the extent of LLC metastasis correlated positively with the severity of bone loss in mice. The metastasis of LLC is accompanied by elevations in plasma concentrations of proinflammatory cytokines, e.g. MCP-1, plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-α (TNF-α) [25, 27]. Available studies show that proinflammatory cytokines participate in bone homeostasis. For example, PAI-1 deficiency attenuates bone loss in estrogen-deficient [28] and diabetic mice [29] and in LLC-bearing mice [24]; TNF-α promotes osteoclastogenesis and osteoclast activation [30, 31]. While exact mechanisms through which LLC reduces bone mass remain to be elucidated, our results indicate that increases in production of proinflammatory cytokines may contribute to bone loss.

This study provides evidence that MCP-1 contributes positively to the bone loss associated with cancer metastasis. This finding is supported by reports that MCP-1 is highly expressed at sites of osteoporotic bone [11], prostate cancer-induced bone resorption [32] and bacteria-induced bone loss [33]. Furthermore, *in vitro* studies show that MCP-1 stimulates osteoclast formation [12, 34] and that lack of MCP-1 decreases the numbers and activity of osteoclasts *in vitro* and elevates bone mass in mice [35].

Adipose tissue, considered an endocrine organ, produces inflammatory cytokines. In the present study, the high-fat diet increased body adiposity and plasma concentrations of all proinflammatory cytokines quantified including MCP-1 (not in MCP-1$^{-/-}$ mice), PAI-1, TNF-α and leptin [25]. As aforementioned, these cytokines participate in bone metabolism, and their elevations often result in bone loss [28-31, 35] and osteoclastogenesis [30, 31]. Thus, elevations of these cytokines likely contributed to the high-fat diet exacerbating bone loss in LLC-bearing mice.

Bone remodeling is a highly coordinated process of bone formation and bone resorption. Osteocalcin is considered an indicator of bone formation [36, 37]. Serum levels of osteocalcin are lower in lung cancer patients with bone metastasis compared to those with no or delayed metastasis [38, 39]. Mounting evidence show that TRAP 5b is a useful marker of bone resorption [40, 41]. Serum concentrations of TRAP 5b correlate positively with the extent of bone lesions in patients with multiple myeloma [42] and increase in breast cancer patients with skeletal metastasis [43]. In the present study, the decrease in plasma osteocalcin and the increase in TRAP 5b in LLC-bearing mice indicate that LLC metastasis may uncouple bone remodeling. This uncoupling may be responsible for the detrimental changes in bone structure and mineral density in those mice.

In LLC-bearing mice, neither the high-fat diet decreased nor MCP-1 deficiency increased the plasma

Table 4: Cortical structural changes in femurs and vertebrae of MCP-1$^{-/-}$ and wild-type mice fed the AIN93G or the high-fat diet

	AIN93G Wild-type No LLC	AIN93G Wild-type	AIN93G MCP-1$^{-/-}$	High-fat Wild-type	High-fat MCP-1$^{-/-}$	*p* values Diet	*p* values Gene	*p* values D × G
Femurs								
Ct.Ar/Tt.Ar, %	47.1 ± 0.6 *	45.4 ± 0.6	45.7 ± 0.4	44.6 ± 0.5	46.5 ± 0.4	0.99	< 0.05	0.10
Ct. Th, µm	165.4 ± 2.2 *	154.6 2.7	153.4 ± 1.9	154.7 ± 1.5	156.1 ± 3.0	0.54	0.98	0.58
Vertebrae								
Ct.Ar/T.Ar, %	76.2 ± 0.7 *	71.8 ± 1.4	73.2 ± 0.8	72.3 ± 0.9	71.5 ± 1.0	0.55	0.76	0.27
Ct.Th, µm	47.1 ± 0.9	42.7 ± 0.9	43.6 ± 0.9	42.6 ± 0.6	43.4 ± 0.6	0.86	0.29	0.93

Two-way ANOVA was performed to compare differences among the groups of LLC-bearing mice. *A priori* contrasts were performed to compare differences between AIN93G-fed wild-type mice with or without Lewis lung carcinoma (LLC); *p<0.01 compared to AIN93G wild-type. Values are means ± SEM (n = 14-15 per group). Ct.Ar/Tt.Ar: cortical area fraction; Ct.Th: cortical thickness.

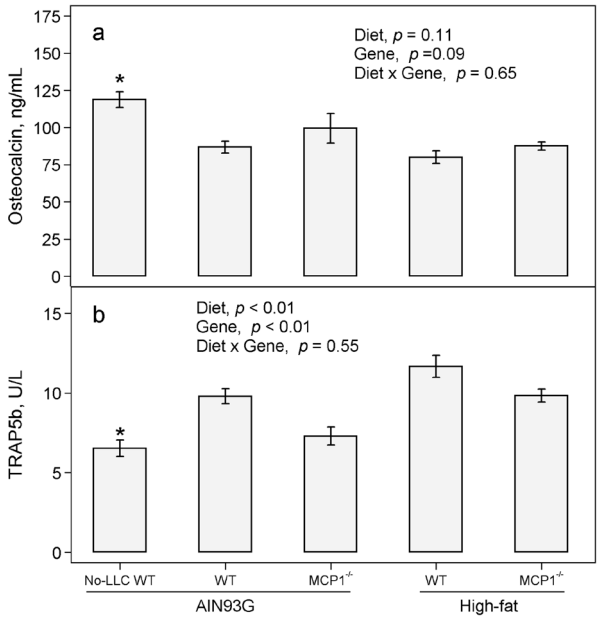

Figure 2: Plasma concentrations of osteocalcin and TRAP 5b in MCP-1$^{-/-}$ and wild-type mice fed the AIN93G or the high-fat diet. Two-way ANOVA were performed to compare differences among the groups of LLC-bearing mice. *A priori* contrasts were performed to compare differences between AIN93G-fed wild-type (WT) mice with or without Lewis lung carcinoma (No-LLC); *p<0.05 compared to AIN93G WT. Values are means ± SEM (n = 12 per group).

concentrations of osteocalcin compared to their corresponding controls. Therefore, their observed effects on bone structure likely were not via bone formation. The finding that the high-fat diet increased plasma TRAP 5b suggests that the high-fat diet may uncouple bone remodeling by promoting bone resorption. This suggestion is supported by reports that elevated TRAP 5b in plasma [22, 44] is associated with obesity-induced bone loss. Our finding that MCP-1$^{-/-}$ mice exhibited decreased plasma TRAP 5b suggests that MCP-1 deficiency may restore bone mass by reducing bone resorption. Furthermore, MCP-1 binds to the MCP-1 receptor expressed in osteoclasts [12] and deficiency in MCP-1 receptor results in higher bone mass in mice [45]. Thus, all these studies support our finding that MCP-1 may contribute to bone loss during LLC metastasis by promoting bone resorption but not by reducing bone formation.

In summary, the present study showed detrimental structural changes in trabecular and cortical bone and reduction in bone mineral density in mice with LLC and its pulmonary metastases. It indicates that LLC aggression leads to bone loss in this mouse model. Our findings support the clinical observations that cancer progression accompanies bone wasting [1] and disturbance of bone remodeling [38, 39, 42, 43]. Furthermore, it indicates the relevance and usefulness of this spontaneous metastasis model to study cancer-associated bone wasting. That feeding mice the high-fat diet exacerbated and MCP-1 deficiency attenuated LLC-mediated bone loss indicate that both high-fat diet and MCP-1 contribute positively to bone loss in metastasis-associated wasting. It suggests that approaches that lead to a reduction in cancer-promoting proinflammatory cytokine MCP-1, including that produced by the adipose tissue, may attenuate not only cancer progression but also its associated bone wasting.

MATERIALS AND METHODS

Animals and diets

Four to five-week old male MCP-1 deficient mice (MCP-1 $^{-/-}$, B6.129S4-*Ccl2*^{tm1Rol}/J, C57BL/6J background) and C57BL/6J wild-type mice (The Jackson Laboratory, Bar Harbor, ME, USA) were housed in a pathogen-free room with a 12:12-hour light/dark cycle and maintained at 22 ± 1°C [25]. Mice were fed a modified AIN93G diet [46] providing 16% or 45% (high-fat diet) of energy from corn oil. Mice had free access to their diets and deionized water; they were weighed weekly. Both diets were powder diets; they were stored at -20°C until feeding.

Lewis lung carcinoma cells

Lewis lung carcinoma cell line, a variant that metastasizes to lungs [26], was obtained from Dr. Pnina Brodt, McGill University, Montreal, Quebec, Canada. The cells were cultured with RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum and maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells used for metastasis studies were *in vivo*-selected once [47]. Cells were free of mycoplasma based on Hoechst DNA staining and direct culture tests performed by American Type Cell Collection (Manassas, VA, USA).

Experimental design

The study was approved by the Institutional Animal Care and Use Committee of the U.S. Department of Agriculture, Agricultural Research Service, Grand Forks Human Nutrition Research Center. The procedures followed the guidelines of the National Institutes of Health for the care and use of laboratory animals [48]. The experimental design was reported previously [25]. Briefly, MCP-1$^{-/-}$ (n = 21 per group) and wild-type mice (n = 28 per group) were fed their respective AIN93G or high-fat diet for 7 weeks before each mouse was subcutaneously injected with 2.5 x 10^5 viable LLC cells in the lower dorsal region. In addition, a separate group of wild-type mice (n = 18) fed the AIN93G diet but not injected with LLC cells served as controls to compare changes in bone structure and related biomarkers to LLC-bearing wild-type mice fed the same AIN93G diet. The subcutaneous primary tumor was removed surgically 11 days later when it was approximately 1 cm in diameter. Following surgery, mice were maintained on their respective diets for an additional 10 days. At termination, mice were euthanized, and their lungs were removed for determination of the extent of metastasis [25]. The right femur and vertebral column (from 10th or 11th thoracic vertebra to sacrum) from each mouse was collected and stored in phosphate-buffered saline for micro-computed tomographic analysis of trabecular and cortical bone. Plasma was collected for quantifying osteocalcin and tartrate-resistant acid phosphatase 5b (TRAP 5b).

Bone evaluation

Femurs were cleaned with cheese cloth for physical measurements before micro-computed tomographic analysis. Femoral length along the proximal distal direction and mid-shaft widths in both medial-lateral and anterior-posterior axes were measured by using a digital caliper (Fred V. Fowler Company, Newton, MA, USA). Femurs and lumbar vertebral bodies were evaluated for trabecular and cortical structural properties by using high-resolution (12-µm slice increment) micro-computed tomography (µCT-40; Scanco Medical, Basserdorf, Switzerland) with *x*-ray source power of 55 KeV and 145 µA and integration time of 300 ms. A fixed threshold of 275 was used to delineate mineralized bone from soft tissue and marrow phase. In distal femurs, trabecular bone was evaluated in 125 slices (1.5 mm) of the metaphysis proximal to the distal growth plate, and cortical bone was evaluated in 100 slices (1.2 mm) at mid-shaft of the femur. In vertebral bodies, trabecular and cortical bone were analyzed along the entire cranial-caudal axis of the 4th lumbar vertebra. The evaluation followed guidelines for assessment of bone microstructure in rodents using micro-computed tomography [49].

For trabecular bone, total volume (TV, mm^3), bone volume (BV, mm^3), bone volume fraction (ratio of the segmented bone volume to the total volume of the region evaluated, BV/TV, %), connectivity density (a degree of connectivity of trabeculae normalized by TV, Conn.D, 1/mm^3), structure model index (an indicator of the plate- and rod-like geometry of trabecular structure, SMI), trabecular number (the average number of trabeculae per unit length, Tb.N, 1/mm), trabecular thickness (mean thickness of trabeculae, Tb.Th, mm), trabecular separation (mean distance between trabeculae, Tb.Sp, mm) and bone mineral density (BMD, mg hydroxyapatite/cm^3) were measured in distal femurs and vertebrae. For cortical bone, total cross-sectional area inside the periosteal envelope (Tt.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), cortical area fraction (Ct.Ar/T.Ar, %) and average cortical thickness (Ct.Th, mm) were computed for the femoral mid-shaft and vertebrae.

Quantification of osteocalcin and TRAP 5b in plasma

Concentrations of osteocalcin (Alpco Diagnostics, Salem, NH, USA) and TRAP 5b (Immunodiagnostic Systems, Scottsdale, AZ, USA) in plasma were quantified by using sandwich enzyme-linked immunosorbent assay kits following protocols provided by the manufacturers. Samples were read within the linear range of the assay; the accuracy of the analysis was confirmed by the controls provided in each assay kit.

Statistical analyses

The effects of diet (AIN93G or high-fat), genotype (MCP-1$^{-/-}$ or wild-type) and their interaction were tested by using two-way analysis of variance (ANOVA) and Tukey contrasts. To examine the effect of LLC on bone structural changes and plasma concentrations of osteocalcin and TRAP 5b, *a priori* contrasts were used to test for differences in AIN93G-fed wild-type mice with or without LLC. Pearson correlation analysis was performed to examine associations between the extent of lung metastasis and trabecular structural changes in femurs and vertebrae. All data are presented as means ± standard error of the mean (SEM). Differences with a p-value of 0.05 or less were considered significant. All analyses were performed by using SAS software (version 9.4, SAS Institute, Cary, NC, USA).

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CONFLICTS OF INTEREST

The authors have declared that no competing interests exist.

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NOTES

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