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Tutorial

Altered bone microarchitecture and gene expression profile due to calcium deficiency in a mouse model of myeloma

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ABSTRACT

It is not clear why patients with an indolent form of multiple myeloma (MM) develop into an aggressive form with poor prognostic. We investigated the effect of a dietary calcium deficiency on tumor growth, osteolysis and gene expression in the 5T2MM murine model.

Two groups of C57BL/KaLwRij mice received 5T2MM cells and started a diet with normal (0.8%; "normal-Ca-MM") or low calcium content (0.05%; "low-Ca-MM"). Two control groups (without 5T2MM cells) received either a normal or low calcium diet (normal-Ca and low-Ca groups). Tumor growth, osteolysis and marrow gene expression of the Wnt pathway, RANKL and MIP-1α were monitored at 6, 8 and 10 weeks (w) after cell injection.

In low-Ca mice, serum level of PTH was higher after 10 w; microCT showed trabecular bone loss and decrease of cortical thickness at the tibia. A higher M-protein level was evidenced at 10 w and 4 mice developed paraplegia at 8/9 w in low-Ca-MM group only. Numerous cortical perforations of the tibia were observed in MM groups with a marked decrease in cortical thickness in low-Ca-MM. At 6 w, osteoclast number from the endosteum was significantly higher in low-Ca-MM compared to normal-Ca MM. This observation was not found at 8 and 10 w. MicroCT of the lumbar vertebrae showed dramatic bone destruction in the low-Ca-MM group. qPCR revealed no difference in RANKL expression whereas differences were obtained in the expression of Lrp5/Lrp6 and MIP-1α from 6 w.

A low calcium diet induced higher bone destruction in the tibia and vertebra associated with an earlier decrease in bone formation level and a higher increase in bone resorption level at early time in the MM development.

1. Introduction

Multiple myeloma (MM) is a hematological malignancy characterized by proliferation of malignant plasma cells in the bone marrow. Osteolysis is one of the main clinical consequences of MM and concerns 90% of the patients. Osteolytic foci in MM patients induce serious clinical manifestations such as bone pain, fractures of long bones, vertebra and/or hypercalcemia. Bone fractures occur in 60% of patients (Roodman, 2009). Moreover, bone fractures have a higher incidence in MM than in other cancer (breast, prostate and lung cancer) with a high frequency of bones metastases. Pathological fractures in MM are associated with a 20% increased risk of death (Saad et al., 2007). In the last decade, the median survival of MM patients has been improved due to the apparition of new treatments. The median survival is now approximately 5–8 years and reaches 10 years for patients with low-risk cytogenetic abnormalities (Avet-Loiseau et al., 2012; Chesi and Bergsagel, 2015). Bone resorption, due to an increase in osteoclast number, is increased in MM patients in the vicinity of plasma cell nodules (Bataille et al., 1989). Secretion of numerous cytokines by malignant plasma cells and the bone marrow microenvironment are responsible for an increased osteoclastogenesis in MM (Roodman, 2009). Several cytokines have been identified to play a key role in osteolysis: tumor necrosis factor alpha (TNFα, interleukin-1β (IL-1β)), interleukin-6 (IL-6), macrophage inflammatory protein-1-alpha and beta (MIP-1α and MIP-1β) and ligand for receptor activator of nuclear transcription factor-κB (RANKL) (Kato et al., 2002; Xi et al., 2016). In overt MM, a decrease in bone formation is associated, leading to an uncoupling in bone remodeling at the later stages of the disease. Inhibition of osteoblastogenesis is due to inhibitors released by plasma cells that depress the Wnt-signaling pathway: DKK1 (Dickkopf-1) and Sfrp2 (Secreted frizzled-related

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protein 2) (Tian et al., 2003) or act on osteoblastic precursors: hepatocyte growth factor (HGF), interleukin-3 (IL-3) and interleukin-7 (IL-7) (Ehrlich et al., 2005; Standal et al., 2007).

Animal models can help to understand the molecular, cellular and tissue aspects of the pathophysiology of human diseases and to develop new therapeutic strategies. Several murine models of human MM have been described in the literature (see reviews in (Libouban, 2015; Paton-Hough et al., 2015)). Among them, the 5T2MM murine model was described in the C57BL/KaLwRij strain (Radl et al., 1988, 1979). It presents the most common aspects of the human disease with moderate growth, homing restricted to the bone marrow microenvironment and osteolytic lesions. A monoclonal protein (M-protein) is detected from 6 weeks (w) after injection and osteolytic lesions are radiographically visible at 8 w (Libouban et al., 2004). The 5T2MM model was extensively used to evaluate the effect of antineoplastic and antiangiogenic drugs (Deleu et al., 2009a; Deleu et al., 2009b) or inhibitors of bone resorption such as bisphosphonates (Croucher et al., 2003; N'Diaye et al., 2015; Radl et al., 1985). The model is also of the utpmost importance to elucidate the pathophysiology of the disease (Buckle et al., 2012; Vanderkerken et al., 2000). It has been shown that a high bone remodeling level (as induced by ovariectomy in mice) accelerates plasma cell growth (Libouban et al., 2003). Similarly, other factors known to increase bone remodeling have also been found to burst the growth of carcinomatous cells (Hirbe et al., 2007; Ooi et al., 2010; Zheng et al., 2008, 2007, 2011). A calcium deficient diet stimulates the growth of MCF-7 human breast cancer cells in the mouse by inducing a secondary hyperparathyroidism (Zheng et al., 2007). More recently, it was shown that a vitamin D deficiency promotes MDA-MB-231 breast cancer cells in the mouse by also inducing a secondary hyperparathyroidism (Ooi et al., 2010). These studies highlight the importance of calcium intake and bone remodeling on tumor growth and osteolysis. They have analyzed the pathophysiological mechanisms at the cell and tissue levels in animal models of bone metastasis; however, molecular alterations in the bone microenvironment have received little consideration. The aim of the present study was to investigate the effect of a dietary calcium deficiency on MM development at the tissue, cell and molecular levels in the 5TMM model. Microcomputed tomography (microCT), histomorphometry and gene expression profile of the microenvironment were used.

2. Material and methods

2.1. The multiple myeloma cell line

We have used a 5T2MM cell line (subclone 5THL) in C57BL/KaLwRij mice as previously characterized (Libouban et al., 2004). These malignant plasma cells can be propagated into young syngeneic mice by intravenous transfer of cells from invaded bone marrow. Progression of the disease in recipient mice was assessed by measuring the serum M-protein level. Around 6 w post-injection, mice had a detectable serum M-protein and were sacrificed after 10–11 w by cervical dislocation. Femurs and tibias were dissected, cleaned of surrounding tissues and bone marrow was flushed in Dulbecco's modified essential medium (DMEM.mod., GIBCO, Life Technologies, France) supplemented with penicillin-streptomycin, amphotericin-fungizone and pyruvate (GIBCO). Bone marrow cells were washed once in DMEM.mod. Mononuclear cells were isolated by a Lympholyte-M centrifugation gradient (Cedarlane, Hornby, Ontario, Canada) at 450 g for 25 min. Mononuclear cells were then washed twice and counted. The recipient mice received 1.5×10^6 cells in the tail vein.

2.2. Animals and study design

Seventy-six C57BL/KaLwRij female mice 6–8-w-old were used for the full study (Harlan, Gannat, France). They were acclimated for one w to the local vivarium conditions (24 °C and 12 h/12 h light dark cycle) where they were given standard laboratory food (UAR, Villemoison sur Orge, France) and water *ad libitum*. The animals were housed at the animal facilities of Angers University Medical School (SCAHU). All procedures have been approved by the Institutional Animal Care and Use Committee at the University of Angers (Agreement number 49028). All procedures were done in accordance with the 2013 French animal procedure act and under the supervision of authorized investigators.

Two experimental procedures were conducted. The first one examined the modification of bone remodeling by dietary calcium deficiency in control animals and used 20 C57BL/KaLwRij mice. The second experimental procedure combined the calcium deficiency and the injection of 5T2MM cells in mice and used 56 C57BL/KaLwRij mice. The two experimental procedures were described in details below.

2.2.1. Modification of bone remodeling by dietary calcium deficiency

On day 0, twenty mice were randomized into 2 groups (10 animals per group). The first group was given standard laboratory chow with normal calcium content (0.8%); they constituted the Normal-Ca group. The second group was given a calcium deficient laboratory chow (0.05% – SAFE AUGY, Augy, France); they constituted the Low-Ca group. At 10 w, blood samples were collected by intracardiac puncture to measure serum parathyroid hormone (PTH) level and mice were then sacrificed by cervical dislocation.

2.2.2. Combined model (dietary calcium deficiency + 5T2MM cells)

Fifty mice were injected with 5T2MM cells in the tail vein and immediately placed on the normal or low calcium diet as above. They constituted respectively the normal-Ca-MM group (n = 25) and the low-Ca-MM group (n = 25). Each group of mice was divided into 3 subgroups to perform a time-dependent analysis of osteolysis by histomorphometry and quantitative PCR (qPCR). Mice were sacrificed by cervical dislocation at 6, 8 and 10 w. Mice, which developed paraplegia before the end of each time point, were sacrificed for ethical reason. Six additional mice were sacrificed at the beginning of the study at w0 and were used as control (control-w0 group) for biochemical and histomorphometric analysis.

2.3. Bone tissue collection

Right femurs and tibias from all mice were dissected, cleaned of soft tissue, and conserved in an ethanol based fixative +4 °C for further analysis. Lumbar vertebrae from L2 to L4 were also dissected for mice in experimental procedure 2, slightly cleaned of soft tissue and conserved in an ethanol based fixative for further analysis. Left femurs of mice, in the experimental procedure 2, were used for qPCR analysis.

2.4. Measurement of the M-protein level in the serum

Progression of MM was monitored by measuring the serum M-protein (IgG2aκ) concentration using agar electrophoresis (Hydragel protéine, SEBIA, Issy les Moulineaux, France). The level of M-protein was expressed as the percentage of the γ-peak using image analysis (NIH image for Windows) on the electrophoretic diagram. Blood was collected from the tail vein at 6 w for all 5T2MM groups to check the presence of the M-protein. For kinetic evolution of the M-protein level, blood was also collected at 8 and 10 w