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Plasma cells release membrane microparticles in a mouse model of multiple myeloma

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ABSTRACT

Microparticles (MPs) released from the plasma membrane play a role in tumor progression. Involvement of MPs in myeloma (MM) has been poorly investigated. Because of the strong interaction of MM cells with bone microenvironment, we hypothesized an implication of MPs in MM using a murine model. Forty-four mice were injected with 5THL-MM cells and compared with 14 non-injected mice. Blood was collected at the early and end stages of MM development (EMM and LMM) to characterize the circulating MPs. At LMM, MPs were isolated from bone marrow (BM) of long bones of 22 mice, after centrifugation. Electron microscopy immunohistochemistry and Western blotting using CD138 were performed on BM-derived MPs. At EMM, MPs circulating level was significantly lower versus controls. In LMM, a significant increase of the total MP number from plasma was observed versus controls. Characterization of circulating MPs showed an increase of leukocyte- and erythrocyte-derived MPs. In LMM, serum M-protein was correlated with circulating MP number. BM-derived MPs increased in LMM and expressed CD138. Anti-CD138 coupled with nanobeads localized at the MP surface. There is evidence of an association between increase of MPs and MM development; the results underscore the participation of plasma cell-derived MPs originating from BM.

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1. Introduction

Multiple myeloma (MM) is a B cell malignancy characterized by monoclonal proliferation of plasma cells in the local bone marrow (BM) environment and the development of osteolytic bone lesions. The BM microenvironment has a key role in MM as it includes a large spectrum of cellular and molecular components that influence plasma cells growth and osteolysis (Asosingh et al., 2003; Lemaire et al., 2011). A true "cious circle" exists where plasma cells stimulate bone and medullar cells which stimulate in return the neoplastic growth. Demonstration that the growth of malignant plasma cells is accelerated with a pre-existing high level of bone remodeling has been shown using a combined animal model in which ovariectomy was associated to injection of 5T2MM plasma cells in the C57BL/KaLwRij mouse (Libouban et al., 2003). Osteoclasts respond to a variety of cytokines and growth factors produced by cells of the BM microenvironment and by the malignant plasma cells. These factors include macrophage inflammatory protein-1-alpha (MIP-1α), the ligand for receptor activator of nuclear transcription factor-κB (RANKL) and interleukin-3 (IL-3) (Asosingh et al., 2003; Choi et al., 2000; Lee et al., 2004; Pearse et al., 2001). It is now well recognized that a decrease of bone formation plays also a key role in MM. Inhibition of osteoblastogenesis is due to osteoblast inhibitors released by plasma cells including Wnt-signaling inhibitors Dickkopf-1 (DKK1) and Secreted frizzled-related protein 2 (Sfrp2), hepatocyte growth factor (HGF) and IL-7 (Giuliani et al., 2005; Oshima et al., 2005; Standal et al., 2007; Tian et al., 2003). Cells involved in the "vicious circle" are of hematological origin at various differentiation stages, BM stromal cells, endothelial and bone cells (osteoblasts and osteoclasts) (Mitsiades et al., 2007). Interaction with endothelial cells mainly supports tumor growth and neoangiogenesis is observed in MM (Roccaro et al., 2006). More recently, interactions with T- and dendritic cells (Giuliani et al., 2006; Kukreja et al., 2006) were revealed thus amplifying the "vicious circle". Thus, MM appears as an excellent model for studying tumor-microenvironment. Implication of the microenvironment interactions in the development of a tumor has been shown in other type of cancers.

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Recently, another mechanism has been highlighted in inducing a favorable local microenvironment for tumor growth: membrane-derived vesicles (MPs) shed from different cell types (Peinado et al., 2011). MPs are membrane-derived vesicles (diameter ≤ 1 μm) that are released during cell activation or apoptosis. On their surface, MPs bear antigens characteristic of the cell of origin, and carry other membrane and cytoplasmic constituents. MPs are present in blood from healthy and non-healthy individuals leading to the hypothesis that they may play physiological and/or pathophysiological roles. Previous studies have revealed various roles of MPs in cancer. It has been recently shown that MPs can induce in vitro neo-angiogenesis which might contribute to the generation of a vascular network in malignant disease associated with tumor growth (Soleti et al., 2009). In addition, MPs released from tumor cells have a pro-angiogenic activity (Kim et al., 2002). Other membrane compounds linked to MPs, such as urokinase, can increase the invasive capacity of prostate cancer cells (Angelucci et al., 2000). MPs are able to carry intercellular signals allowing the tumor survival and progression. Considering that enhanced levels of circulating MPs have been detected in patients with different types of cancers, one can advanced the hypothesis that MPs might play an important role in the tumor development (Martinez and Andriantsitohaina, 2011; Mostefai et al., 2008; Tual-Chalot et al., 2011).

Because of the various cell types involved in MM and the strong interaction of the bone and medullar microenvironment, we hypothesized an implication of MPs in the development of MM. In the present study, quantification and characterization of both circulating-MPs and BM-derived MPs were investigated in the 5THL murine model of MM.

2. Materials and methods

2.1. Mice

C57BL/KaLwRij female mice (6–8-week-old) were used for the study (Harlan, Gannat, France). They were acclimated for 1 week to the local vivarium conditions (24 °C and 12 h/12 h light/dark cycle) where they were given standard laboratory food (UAR, Villemoisson sur Orge, France) and water ad libitum. The Animal Care and Use committee at the University of Angers approved all procedures.

2.2. Culture cell line

We have used the 5THL cell line as previously characterized (Libouban et al., 2004). Briefly, the 5THL cell line is an aggressive subline of 5T2MM cell line originating from elderly C57BL/KaLwRij mice that spontaneously developed MM (Croese et al., 1987). 5THL cells can be propagated into young syngeneic mice by intravenous transfer of the diseased BM. Progression of the disease in seven recipient mice was assessed by measuring the serum M-protein (IgG2aκ) level using agar electrophoresis (Hydragel Protein, SEBIA, Issy les Moulineaux, France). Around 6 week post-injection of 5THL, mice had a detectable serum M-protein and were euthanized after 10–12 weeks by cervical dislocation. Femurs and tibias were dissected, cleaned of surrounding tissues and BM was flushed in Dulbecco's modified essential medium (DMEM.mod, GIBCO, Life Technologies, France) supplemented with penicillin–streptomycin, amphotericin–fungizone and pyruvate. BM cells were washed once in DMEM.mod. Mononuclear cells were isolated by a Lympholyte-M centrifugation gradient (Cedarlane, Hornby, Ontario, Canada) at 1250 × *g* for 20 min. Mononuclear cells were then washed twice in DMEM.mod and counted.

2.3. Experimental design

Forty-four mice (6–8 weeks old) were injected with 1.5×10^6 5THL cells in the tail vein and 14 non-injected mice were used as control (CTL). The injected mice were divided in 2 groups according to the time of sacrifice. At 6 weeks, corresponding to the early stage of MM development, 14 mice were bled before being sacrificed by cervical dislocation. These mice constituted the early stage MM group (EMM) and were used to quantify and characterize the phenotype of circulating MPs. At the end stage of the disease (10–12 weeks), when osteolysis can be evidenced on X-ray images, the remaining 30 mice were bled before being sacrificed by cervical dislocation. These mice constituted the late stage MM group (LMM) and were used to quantify and characterize the phenotype of circulating MPs (see below). Twenty-two mice from the LMM group were also used to isolate MPs from the BM of femurs and tibias. The 14 non-injected mice (16–18-week-old) were bled as previously described to quantify and phenotype circulating MPs. Eight of the 14 mice were also used to isolate MPs from BM.

Two additional mice injected with 5THL cells were sacrificed at the end stage of the disease and were used for transmission electron microscopy (TEM) observations and immunohistochemistry of BM-derived MPs and MM cells.

2.4. Circulating MP isolation and characterization

C57BL/KaLwRij mice at 6 weeks or at 10–12 weeks were anesthetized using Isoflurane (Baxter, Maurepas, France) before sacrifice, and ~800 μl of blood were collected by aspiration from the left ventricle. Blood was placed in citrated tubes and centrifuged at 1900 × *g* for 3 min for separation of platelet-rich plasma from whole blood. Then, platelet-rich plasma was centrifuged at 5000 × *g* for 4 min to obtain platelet-free plasma (PFP). Sixty microliters of PFP were frozen and stored at −80 °C until subsequent use. In order to pellet MPs for in vitro studies, circulating MPs were concentrated from PFP by three series of centrifugations at 21,000 × *g* for 45 min and re-suspended in saline and stored at 4 °C until subsequent use.

Membrane MP subpopulations were discriminated in PFP according the expression of membrane-specific antigens. Phenotype of endothelial MPs was performed using anti-CD54 labeling; characterization of platelet, leukocyte and erythrocyte MPs was performed using respectively anti-CD61, anti-CD45 and Ter-119/erythroid cell labeling. Irrelevant mouse IgG was used as an isotype-matched negative control for each sample.

For numeration studies, 8 μl of PFP were incubated with either 1 μl of specific antibody (BioLegend, San Diego, CA). After 45 min of incubation at room temperature, samples were diluted in 300 μl of saline. Annexin V (BioVision, Inc., Mountain View, CA) binding was used to numerate circulating phosphatidylserine-expressing MPs (2 μl of annexin V/5 μl PFP). Then, in order to enumerate MPs, an equal volume of sample and Flowcount beads were added and samples were analyzed in a flow cytometer 500 MPL system (Beckman Coulter, Roissy, France) as previously described (Agouni et al., 2008; Mostefai et al., 2008). Flow-count fluorospheres consist of 10 μm polystyrene fluorospheres in an aqueous suspension medium. Each fluorosphere contains a dye that has a fluorescence emission range of 525–700 nm when excitated at 488 nm. The concentration of each lot of Flow-count fluorospheres is derived from multiple replicate analyses on a COULTER particle size analyzer performed by the manufacturer. Each lot of Flow-count fluorospheres has a specific concentration of fluorospheres. When identical volumes of a sample and Flow-count fluorospheres are used, a ratio of MPs in the sample to fluorospheres is established. Since the concentration of fluorospheres is known, the absolute count of the MPs can be automatically determined by the MXP software.

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