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Contact of myeloma cells induces a characteristic transcriptome signature in skeletal precursor cells –Implications for myeloma bone disease

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ABSTRACT

Physical interaction of skeletal precursors with multiple myeloma cells has been shown to suppress their osteogenic potential while favoring their tumor-promoting features. Although several transcriptome analyses of myeloma patient-derived mesenchymal stem cells have displayed differences compared to their healthy counterparts, these analyses insufficiently reflect the signatures mediated by tumor cell contact, vary due to different methodologies, and lack results in lineage-committed precursors.

To determine tumor cell contact-mediated changes on skeletal precursors, we performed transcriptome analyses of mesenchymal stem cells and osteogenic precursor cells cultured in contact with the myeloma cell line INA-6. Comparative analyses confirmed dysregulation of genes which code for known disease-relevant factors and additionally revealed upregulation of genes that are associated with plasma cell homing, adhesion, osteoclastogenesis, and angiogenesis. Osteoclast-derived coupling factors, a dysregulated adipogenic potential, and an imbalance in favor of anti-anabolic factors may play a role in the hampered osteoblast differentiation potential of mesenchymal stem cells. *Angiopoietin-Like 4* (*ANGPTL4*) was selected from a list of differentially expressed genes as a myeloma cell contact-dependent target in skeletal precursor cells which warranted further functional analyses. Adhesion assays with full-length ANGPTL4-coated plates revealed a potential role of this protein in INA-6 cell attachment.

This study expands knowledge of the myeloma cell contact-induced signature in the stromal compartment of myelomatous bones and thus offers potential targets that may allow detection and treatment of myeloma bone disease at an early stage.

Abbreviations: ABCA1, ATP-binding cassette, sub-family A (ABC1) member 1; ALP, alkaline phosphatase; ANGPTL4, angiopoietin-like 4; BSA, bovine serum albumin; CA9, carbonic anhydrase IX; C/EBP, CCAAT/enhancer binding protein; CMFDA, 5-chloromethylfluorescein diacetate; DKK-1, dickkopf-1; EEF1A1, eukaryotic translation elongation factor 1 alpha 1; FACS, fluorescence activated cell sorting; FCS, fetal calf serum; FC, fold change; FIGN, fidgetin; HBSS, hank's buffered salt solution; HILPDA, hypoxia inducible lipid droplet-associated; IL-6, interleukin-6; ITPR, inositol 1,4,5-trisphosphate receptor; JAM2, junctional adhesion molecule 2; KISS1R, Kiss1 receptor; LFA-1, leukocyte function-associated antigen-1; MACS, magnetic activated cell sorting; MEOX2, mesenchyme homeobox 2; MGUS, monoclonal gammopathy of undetermined significance; MIR210HG, MIR210 host gene; MM, multiple myeloma; MSC, mesenchymal stem cells; MM-MSC, MSC from MM patients; NRN1, neuritin 1; OPC, osteogenic precursor cells; PPARγ, proliferator-activated receptor γ; PBMCs, peripheral blood mononuclear cells; PGF, placental growth factor; RANKL, receptor activator of NF-κB ligand; RT, reverse transcriptase; SPAG4, sperm associated antigen 4; T_A, annealing temperature; VEGFA, vascular endothelial growth factor A.

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1. Introduction

Multiple myeloma (MM) is a plasma cell neoplasia that is still largely incurable, although the median 10-year survival has increased to 40% in patients < 50 years of age due to the development of new drugs and bone marrow transplantation strategies during the last two decades [1]. MM is preceded by the non-cancerous plasma cell neoplasm monoclonal gammopathy of undetermined significance (MGUS) [2,3]. Progression to malignancy is driven, among other factors, by changes that occur in the tumor microenvironment leading to enhanced angiogenesis, immunosuppression, and bone resorption [4]. Myeloma bone disease is the result of intensive interaction between myeloma cells and the bone that often precipitates in osteolytic lesions, severe osteoporosis with pathological fractures, and bone pain. Together with other complications such as hypercalcemia and paralysis, myeloma bone disease has a very strong impact on the course of the disease, patient's quality of life, and overall survival. Over long periods it may even be the dominant determining factor for the individual burden and the clinical outcome [5,6].

Myeloma bone disease is the result of disturbed bone remodeling with enhanced osteoclastogenesis and decreased bone formation. The molecular mechanisms have been partly unraveled, in analogy to bone metastases from other solid tumors like breast and prostate cancer. Tumor-derived signaling substances, such as receptor activator of NF-κB ligand (RANKL), directly or indirectly stimulate osteoclast activity causing bone loss and pathological fractures. More recently, it has been demonstrated that myeloma and other tumor cells also produce factors inhibitory for osteogenic differentiation and bone formation, such as inhibitors of the Wnt signaling pathway dickkopf (DKK)-1 and sclerostin, which results in completely abolished anabolic bone metabolism and regeneration [6,7,8]. Besides soluble factors, physical interaction between myeloma and mesenchymal stem cells (MSC) or osteogenic precursor cells (OPC) leads to a hampered osteoblast differentiation of the skeletal precursor cells [9] which can be partially restored by the anti-cancer agent and proteasome inhibitor bortezomib [10].

MSC have been reported to inhibit myeloma progression *via* pro-apoptotic mechanisms [11], but generally, their role in myeloma development and survival is described as a supportive one [12,13,14,15,16]. Comparison of gene expression profiles between MSC from MGUS patients/healthy donors with those from MM patients demonstrate tumor-promoting abnormalities in the latter [17,18]. The support of MM development by MSC may be genuine and intrinsic, *e.g.* as part of genetic predisposition. However, several studies revealed that signature changes of MSC are also acquirable by physical contact with myeloma cells [19] (unpublished results from Herman and colleagues: cited in [18]). Nonetheless, genome-wide analyses of MM cell contact-induced changes in the MSC transcriptome are scarcely published [18,19]. Moreover, to our knowledge, no study has been performed with MSC-derived osteogenic precursors, although it is known that their osteogenic potential is also suppressed by direct interaction with MM cells [9].

Therefore, we performed a thorough transcriptome analysis of both MSC and OPC after direct contact with MM cells. These results show that OPC are also capable of supporting MM progression, albeit to a lesser extent than MSC, which seem to have a greater tumor-promoting potential according to the genetic signature. Besides a general overview of altered signaling pathways that support the importance of bone-forming cells in myeloma bone disease the data indicate that osteogenesis and angiogenesis may be uncoupled at the bone-tumor interface. Furthermore we identified *Angiopoietin-Like 4* (*ANGPTL4*) as a novel interaction-specific target of skeletal precursors that mediates myeloma cell attachment. These data support the potent role of stroma in pathophysiological mechanisms favoring MM bone disease and provide insights into molecular mechanisms and signaling pathways that are associated with the development of bone metastases and niche hijacking in general.

2. Materials and methods

2.1. Ethics statement

Bone material was used in accordance with the local Ethics Committee of the Medical Faculty of the University of Würzburg with written, informed consent of each patient.

2.2. Cells and cell culture

2.2.1. Primary cells and cell lines

Primary human MSC were isolated from the cancellous bone from the acetabulum received from donors after total hip arthroplasty (gender: 11 females, 13 male; average donor age in years: mean ± SD: 63 ± 9; range: 48–77 years). MSC were isolated by surface adherence and characterized as previously described [20,21,22]. Expansion was performed in DMEM/Ham's F-12 (1:1) medium (Life Technologies GmbH, Darmstadt, Germany), supplemented with 10% (v/v) fetal calf serum (FCS) (Biochrom, Berlin, Germany), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies GmbH) and 50 μg/ml L-Ascorbic acid 2-phosphate (Sigma-Aldrich Chemie GmbH (Sigma), Schnelldorf, Germany). MSC were passaged at least once before they were used for experiments.

The plasmacytoma cell line INA-6 [23] was authenticated by DNA profiling using 8 different highly polymorphic short tandem repeat loci (performed at the Leibniz Institute, Braunschweig, Germany). For cultivation, cells were incubated in RPMI 1640 medium (Life Technologies GmbH) supplemented with 20% (v/v) FCS, 100 μg/ml gentamicin, 2 mmol/l L-glutamine (Life Technologies GmbH), 1 mmol/l sodium pyruvate (Sigma), and 2 ng/ml recombinant human interleukin-6 (IL-6; R&D Systems, Wiesbaden, Germany). In case of experiments comprising MSC and OPC, INA-6 cells were washed with PBS and resuspended in the respective propagation medium without IL-6 before used for co-culture studies [24]. OPM-2 [25], MM.1S [26], AMO1 [27], and U266 cells [28] were propagated and cultivated in RPMI1640 medium comprising 10% (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l L-glutamine, and 1 mmol/l sodium pyruvate.

$CD19^+$ B-cells were used as a control for myeloma cell lines as previously published [29]. Primary $CD19^+$ B-cells were purified from peripheral blood mononuclear cells (PBMCs) by magnetic activated cell sorting (MACS) (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). PBMCs were obtained by ficoll density centrifugation of leukocyte concentrate. Leukocyte concentrate from whole blood donation (purchased from the Blood Donation Service of the Bavarian Red Cross, Wiesentheid, Germany) was diluted 1:1 (v/v) by PBS, coated onto Ficoll® Paque Plus (GE Healthcare Europe GmbH, Munich, Germany) in a ratio of 2:1, and centrifuged for 30 min (609*g*, brake off). The interphase was washed with PBS buffer comprising 0.9% (w/v) sodium chloride (AppliChem GmbH, Darmstadt, Germany), 1% (v/v) FCS, and 2 mM EDTA (AppliChem GmbH). Isolation of $CD19^+$ B-cells was performed according to the manufacturer's instructions using CD19 MicroBeads and LS columns (Miltenyi Biotec GmbH). $CD19^+$ B-cells were incubated in RPMI1640 medium, 10% (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l L-glutamine, and 50 μmol/l beta-mercaptoethanol (Life Technologies GmbH) for one day prior to the experiments.

2.2.2. Differentiation of MSC to OPC

For differentiation of MSC to OPC, MSC were incubated for two weeks with DMEM High Glucose medium (Life Technologies GmbH) including 10% (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and additionally supplemented with 10 mmol/l beta-glycerophosphate (Sigma), 100 nmol/l dexamethasone (Sigma), and 50 μg/ml L-Ascorbic acid-2-phosphate (Sigma). Osteogenic differentiation medium and DMEM High Glucose medium (control) were changed every 3 to 4 days. Osteogenic differentiation was demonstrated by staining of alkaline phosphatase (ALP) and mineralized extracellular matrix using