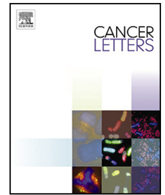
Cancer Letters ■■ (2016) ■■–■■

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet



Original Articles

Adipocytes contribute to the growth and progression of multiple myeloma: Unraveling obesity related differences in adipocyte signaling

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A R T I C L E I N F O

Article history:
Received 12 April 2016
Received in revised form 9 June 2016
Accepted 13 June 2016

Keywords:
Multiple myeloma
Adipocyte
Obesity
Adhesion
Angiogenesis
Adipose-derived stem cells

A B S T R A C T

The prevalence of obesity over the last several decades in the United States has tripled among children and doubled among adults. Obesity increases the incidence and progression of multiple myeloma (MM), yet the molecular mechanisms by which adipocytes contribute to cancer development and patient prognosis have yet to be fully elucidated. Here, we obtained human adipose-derived stem cells (ASCs) from twenty-nine normal (BMI = 20–25 kg/m^2), overweight (25–30 kg/m^2), obese (30–35 kg/m^2), or super obese (35–40 kg/m^2) patients undergoing elective liposuction. Upon differentiation, adipocytes were co-cultured with RPMI-8226 and NCI-H929 MM cell lines. Adipocytes from overweight, obese and super obese patients displayed increased PPAR-gamma, cytochrome C, interleukin-6, and leptin protein levels, and decreased fatty acid synthase protein. 8226 MM cells proliferated faster and displayed increased pSTAT-3/STAT-3 signaling when cultured in adipocyte conditioned media. Further, adipocyte conditioned media from obese and super obese patients significantly increased MM cell adhesion, and conditioned media from overweight, obese and super obese patients enhanced tube formation and expression of matrix metalloproteinase-2. In summary, our data suggest that adipocytes in the MM microenvironment contribute to MM growth and progression and should be further evaluated as a possible therapeutic target.

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Introduction

Multiple myeloma (MM), a plasma cell neoplasia, represents approximately 10% of all hematologic cancers [1]. Despite a dramatic improvement in the average prognosis of MM patients over the last two decades due to more effective drugs and treatment strategies, MM remains an incurable malignancy [2]. In addition to established risk factors such as age, African ancestry, male sex, monoclonal gammopathy of undetermined significance, and family history, in recent years, numerous epidemiological studies have identified obesity as a risk factor for MM [3–6]. Obesity significantly increases both the relative risk of developing MM and MM-associated mortality [3–6]. Despite this epidemiological association, the molecular underpinnings by which obesity contributes to MM growth and progression are relatively unknown.

MM is characterized by clonal expansion of abnormal plasma cells in the bone marrow [7]. The bone marrow microenvironment plays a supportive role in growth, migration, proliferation, survival, and drug resistance of MM cells [8,9]. Within human bone marrow adipocytes are the most abundant cell type [10]. Adipocytes, although traditionally thought of as having functions limited to energy storage, are now considered a major endocrine organ [11]. Adipocytes secrete various adipokines and inflammatory factors and reciprocal signaling between adipocytes and cancer cells is reported to contribute to tumor initiation, growth and metastasis in several types of cancer [11]. Adipocytes from overweight and obese individuals display an altered cytokine and lipid profile when compared to adipocytes from normal weight individuals, as adipocytes from individuals with a higher BMI have increased production of inflammatory markers and leptin and decreased production of anti-inflammatory cytokines and the tumor suppressor adiponectin [12,13]. This exaggerated inflammatory response may increase genomic instability, disturb DNA repair, promote tumor progression, cause local immunosuppression, or induce epigenetic changes [14,15].

In this study we co-cultured human MM cell lines with adipocytes from normal, overweight, obese or super obese patients who received elective liposuction. We found a positive correlation between BMI and adhesion and angiogenesis of MM cells. Moreover, we identified hormonal, lipid, and signaling factor dysregulation in obese adipocytes that can contribute to MM growth and progression.

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http://dx.doi.org/10.1016/j.canlet.2016.06.010
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Please cite this article in press as: Erica M. Bullwinkle, et al., Adipocytes contribute to the growth and progression of multiple myeloma: Unraveling obesity related differences in adipocyte signaling, Cancer Letters (2016), doi: 10.1016/j.canlet.2016.06.010

Materials and methods

Cell lines

Human MM cell lines RPMI 8226 and NCI-H929 (ATCC; Manassas, VA) were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% PenStrep, and 1% GlutaMAX (Life Technologies; Grand Island, NY) at 37 °C and 5% CO_2.

Stem cell isolation

Lipoaspirates from patients undergoing elective liposuction at the office of DaVinci Plastic Surgery (Washington, DC) were used in this study. All patients gave informed consent prior to surgery. Lipoaspirate cells were washed extensively to remove contaminating red blood cells and debris. Washed lipoaspirate samples were then digested with 0.075% Type II Collagenase in HBSS (Life Technologies, Grand Island, NY) for 30–45 at 37 °C. Collagenase was neutralized with HBSS + 10% FBS, and cells were centrifuged at 1000 RPM for 5 minutes, filtered through 100 µm mesh filter, and centrifuged again. Adipose-derived stem cells (ASCs) were plated and extensively washed 24 hours later to remove any residual red blood cells. For some studies additional ASCs were purchased from Zen-Bio, Inc. (Research Triangle Park, NC). All ASCs were used at passage 2. All ASCs and subsequently differentiated adipocytes were grouped into one of four categories: normal (BMI = 20–25 kg/m^2), overweight (25–30 kg/m^2), obese (30–35 kg/m^2), and super obese (35–40 kg/m^2).

Adipocyte, chondrocyte and osteoblast differentiation and detection

ASCs were cultured in subconfluent conditions in supplemented DMEM until they reached passage 2. ASCs were functionally evaluated by inducing differentiation into the following cell types: adipocytes, chondrocytes, and osteoblasts. Adipocyte differentiation media contained 10% FBS, 1% PenStrep, 1.0 µM Dexamethasone, 0.5 mM Isobutylmethylxanthine (IBMX), 0.2 mM Indomethacin, and 10.0 µM insulin, (Sigma-Aldrich; St. Louis, MO). Adipogenesis was visualized on day 17 by Oil Red O staining. Adipocytes were paraformaldehyde-fixed, washed with 60% isopropyl alcohol, incubated in Oil Red Solution (Sigma-Aldrich; St. Louis, MO), and imaged. ASCs differentiated into chondrocytes were plated in 1×10^7 micro-mass 10 µL droplets. Cultures were grown in chondrocyte differentiation media (DMEM supplemented with 1% FBS, 1% PenStrep, 50 µg/mL L-Ascorbic Acid 2-Phosphate, 6.25 µg/mL insulin, 6.25 µg/mL transferrin, and 10 ng/mL TGFβ-1) for 17 days. Mature chondrocytes were washed, fixed, stained with Alcian Blue stain and imaged. ASCs were differentiated into osteoblasts by culturing in DMEM supplemented with 5% FBS, 1% PenStrep, 0.1 µM Dexamethasone, 50 µM L-ascorbic acid 2-phosphate, and 10 mM β-1 glycerophosphate. Mature osteoblasts were stained using Von Kossa Stain. Briefly, cells were fixed, incubated in 2% silver nitrate, exposed to UV light, washed, and imaged.

Adipocyte – MM co-culture

Fully differentiated adipocytes from normal, overweight, obese or super obese patients were cultured alone or co-cultured with RPMI 8226 or NCI-H929 MM cells (ATCC; Manassas, VA) using 0.4 µm polyethylene terephthalate hanging cell culture insert (EMD Millipore; Chicago, IL). Additional blank wells containing no adipocytes were plated with normal supplemented media to be used as controls. After 24 hours of co-culture, conditioned media, protein and RNA were collected from adipocytes (adipocyte conditioned media) or blank wells ("media alone" control). Conditioned media was filtered prior to use to remove cellular debris.

Tube formation assay

1×10^5 3B-11 endothelial cells were serum-starved in DMEM with 0.2% FBS overnight, calcein stained (Life Technologies; Grand Island, NY), plated onto basement membrane extract (BME) as described elsewhere [16], and exposed to pooled conditioned media from normal, overweight, obese or super obese adipocytes. The tube network was allowed to grow for 6–9 hours before paraformaldehyde fixing and then imaged using a fluorescent microscope (Olympus; Center Valley, PA). The tube network (number of nodes/meshes/segments) was quantified using ImageJ Angiogenesis Analyzer Plugin, HUVEC Fluo Analysis.

Western blotting

Total protein lysates were prepared from MM cell lines, MM cells that had been co-cultured with adipocytes and adipocytes alone using M-PER reagent (Thermo Fisher Scientific, Rockford, IL, USA) containing Halt protease/phosphatase inhibitor (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Proteins were quantified using BCA protein assay kit (Thermo Scientific; Waltham, MA) and samples pooled within each BMI category. Proteins were separated using 4–12% Bis-Tris protein gels (Life Technologies; Grand Island, NY). Gels were electrophoresed and transferred to PVDF membrane using the iBlot 2 Dry Blotting System (Life Technologies; Grand Island, NY). Membranes were blocked in 5% BSA. Primary antibodies (Cell Signaling; Danvers, MA) were applied in a 1:1000 dilution in 5% BSA. Anti-rabbit HRP secondary antibody was applied at a dilution of 1:2000 (Cell Signaling Technology). West Dura Chemiluminescent substrate (Thermo, Rockland, Il) was used for signal detection. Membranes were visualized using a ChemiDoc-It imaging system (UVP, Upland, CA). All bands on the Western blot were quantified using NIH ImageJ and normalized to the densitometry for the respective β-actin band. Analysis of variance (ANOVA) results for normalized samples were obtained using StatPlus One-Way ANOVA analysis with group variables. To determine whether individual comparisons (e.g. BMI 25–30 vs. 30–35) were significant, Tukey's LSD post-hoc test was conducted. All Western blots were replicated a minimum of three times.

IL-6 ELISA

IL-6 was measured through an IL-6 ELISA assay (Thermo Fisher Scientific; Grand Island, NY) according to manufacturer's protocol. Briefly, biotinylated antibody was added to a 96 well plate, followed by addition of standards or conditioned media samples from adipocytes. After incubation and washings, streptavidin-HRP was added, plate was incubated, washed, substrate added and absorbance measured at 450 nm. Concentration was determined using a standard curve.

Zymography

To measure MMP-2 enzymatic activity, zymography was conducted on adipocyte conditioned media samples using 10% Tris-Glycine gels containing 0.1% gelatin (Life Technologies; Grand Island, NY). Fifteen micrograms of protein in β-mercaptoethanol-free 2X loading buffer were electrophoresed at 125 V for 90 min in Tris-Glycine/sodium dodecyl sulfate running buffer. After renaturation, developing solution was added and gels were washed, stained using Invitrogen SimplyBlue™ SafeStain (Life Technologies; Grand Island, NY) and destained in deionized water. Densitometry was performed on all bands using NIH ImageJ.

Viability

To assess MM cell viability, WST-1 Reagent was used (Cayman Chemical; Ann Arbor, MI) per manufacturer's instructions. Briefly, 2.5×10^4 MM cells were grown in normal supplemented media or adipocyte-conditioned normal supplemented media in 96-well plates. After 48 hours, 10 µL of Wst-1 Reagent was added to each well, incubated at 37 °C and measurements taken at 450 nm.

Adhesion assay

MM cells were grown in adipocyte conditioned media in 96-well plates. 2×10^4 H929 or 1×10^4 8226 cells were plated in 100 µL of adipocyte conditioned media and grown for 2 days. Non-adherent MM cells were removed and cells were washed and fixed with paraformaldehyde for 15 minutes at room temperature. Images were taken and number of adherent cells was obtained using the NIH ImageJ with Cell Counter Plugin.

Real-time polymerase chain reaction (qPCR)

SuperScript® III Platinum® SYBR® Green Two-Step qPCR Kit w/ROX was used per manufacturer's instructions (Life Technologies; Grand Island, NY) using 1 µg of purified total RNA as template for amplifications. Reverse transcribed cDNA was diluted 1:4 with ultrapure water. qPCR was performed on an Mx3005P QPCR System (Agilent Technologies). Threshold cycle values were calculated with the MxPro QPCR software (v. 4.10) using the Pfaffl method, and dissociate curves for each reaction were checked to confirm that only a single PCR product was obtained [17]. Threshold cycle values were normalized with GAPDH, and relative expression was calculated comparing differences between adipocyte samples. An unpaired t-test between triplicates was used to determine level of significance. All experiments were repeated a minimum of three times. The specific oligonucleotide primers obtained from Life Technologies (Carlsbad, CA) are displayed in Table 1.

Statistical analysis

Viability, qPCR, zymogram, adhesion, and tube formation data were analyzed through analysis of variance (StatPlus One-Way ANOVA). *P* values of 0.05 and below were determined to be significant. To determine whether individual comparisons (e.g. BMI 25–30 vs. 30–35) were significant, Tukey's LSD post-hoc test was conducted.

Results

BMI negatively correlates with ASC viability and positively correlates with markers of differentiation and lipid signaling in adipocytes

Cells from twenty-nine patients undergoing elective liposuction were used in this study (Table 2). Samples from patients were divided into four categories based on BMI of the patient: normal (BMI = 20–25 kg/m^2), overweight (BMI = 25–30 kg/m^2), obese (BMI = 30–35 kg/m^2), and super obese (BMI = 35–40 kg/m^2). Patients

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