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Original Articles

Leptin contributes to long-term stabilization of HIF-1α in cancer cells subjected to oxygen limiting conditions

Alessia Calgani [a], Simona Delle Monache [a], Patrizia Cesare [b], Carlo Vicentini [b], Mauro Bologna [b], Adriano Angelucci [a,*]

[a] Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, 67100 L'Aquila, Italy
[b] Department of Life, Health and Environmental Sciences, University of L'Aquila, 67100 L'Aquila, Italy

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ABSTRACT

Leptin, a cytokine produced by the adipose tissue in response to food intake, is a key player in the regulation of energy balance and body weight control. Physiological action of leptin in modulating the metabolic adaptation of different peripheral tissues supports the hypothesis that it could also exert a direct effect on cancer cells.

In vitro, treatment with leptin up-regulated HIF-1α and stimulated adhesion and invasion of prostate cancer cells cultured in hypoxia. Leptin action was effective in both low and high glycolytic cancer cell lines, and determined the up-regulation of lactate exporter MCT4 and its associated protein CD147. HIF-1α stabilization was oligomycin-independent and was associated with an important modulation of mitochondrial homeostasis. In fact, leptin treatment produced mitochondrial biogenesis, stabilization of mitochondrial membrane potential and increased uncoupled respiration through the up-regulation of UCP2. Furthermore, leptin counteracted the downmodulation of SIRT1 induced by hypoxia, and persistent high levels of SIRT1 were directly involved in HIF-1α stabilization.

Leptin can sustain cancer progression in hypoxic environment and when mitochondrial respiration is impaired. Leptin signaling axis, including the new proposed intermediate SIRT1, could represent a new diagnostic and therapeutic target in prostate cancer.

Introduction

Metabolic adaptation is a hallmark of cancers and its underlying molecular determinants are increasingly recognized [1]. Indeed, cancer cell population shows high capacity to enact effective strategies in sustaining energy requirement associated with unlimited growth. In this context, the best-characterized molecular schemes adopted by cancer cells include the angiogenesis stimulation and the Warburg effect (aerobic glycolysis), both permitting to overcome the restrictive limits posed by fluctuating oxygen tension and paucity of intermediates for biosynthetic reactions [2].

The accumulation, and in turn the transcriptional activity, of hypoxia-inducible factor 1 alpha (HIF-1α) allows a robust induction of the angiogenic switch and glycolysis. HIF-1α upregulation is transiently noticed in hypoxia, a condition frequently associated with cancer growth, also in prostate cancer, and it is a factor allowing the cells to adapt to hypoxic microenvironments already present in the early phases of carcinogenesis [3,4]. Indeed, chronic activation of HIF-1α is a key feature of many cancers and a well-known characteristic also shared in vitro by the majority of cancer cell lines in aerobic conditions. Because of prolonged HIF-1α activity, there is a significant advantage in cancer cells in terms of proliferation, migration and drug resistance; therefore, HIF-1α is now considered an oncoprotein [5].

Endocrine factors may have an indirect accelerating action on carcinogenesis or cancer progression through the modulation of cancer energetic homeostasis [6]. In particular, information about the metabolic effects of circulating factors frequently perturbed in obese men, including sex hormones, insulin, IGF-1, pro-inflammatory cytokines and adipokines, are present in the literature. Androgens have been indicated as in vitro modulators of HIF-1α activation [7]. At the same time systemic pro-inflammatory cytokines, including TNF, can enhance HIF-1α transcription, mainly through NF-κB (nuclear factor kappaB) activity [8].

The association of obesity with cancer is a well-known epidemiologic evidence for many types of cancers. High BMI is associated with nearly 4% of new cancer cases per year worldwide, mainly with those of esophagus, colon, endometrium, kidney and breast; moreover, 14–20% of the total cancer deaths were attributable to obesity in adults older than 50 years [9]. In addition, convincing data have associated obesity with an increased risk of advanced prostate cancer, metastasis or death from prostate cancer [10,11].

* Corresponding author. Tel.: +39 0862 433550; fax: +39 0862433523.
E-mail address: adriano.angelucci@univaq.it (A. Angelucci).

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Adipokines are endocrine peptides released by fat tissue with the main function of regulating energy homeostasis in consequence of variation in fat accumulation. Adipokines have been proposed as the possible link between obesity and prostate cancer progression [12]. Leptin is a small adipokine produced predominantly by adipocytes: its serum levels are higher in obese individuals than in lean subjects. It is increasingly recognized that leptin exerts a complex control of systemic energy homeostasis, targeting different tissues. Leptin receptor (LEPR) has been detected in an increasing number of tissues, including lung, ovaries, testis and placenta, suggesting for leptin the existence of still unexplored peripheral functions [13]. Data about a direct role of leptin on prostate cancer are contradictory and the molecular links with the aggressive phenotype are largely speculative. However, some results from in vitro prostate cancer models suggest that leptin activates specific intracellular signaling pathways associated with proliferation, migration and invasion [14]. It is of note that in an animal model, leptin has demonstrated to be a critical peripheral tumor promoter, independent of the body weight of the animals [15].

In our work, we demonstrate for the first time that leptin can stimulate prostate cancer survival in an adverse metabolic microenvironment by sustaining HIF-1α activity. This association is evident in hypoxic conditions and is independent of oxidative phosphorylation (OXPHOS). These data can contribute to explain the higher occurrence of advanced prostate cancer in obese men with high levels of serum leptin.

Materials & methods

Cell culture

LNCaP, a human prostate cancer cell line isolated from metastatic lymph node (European Collection of Cell Cultures, ECAC, Porton Down, Salisbury, UK #CRL1740, Lot No. 08G024, passage no. 15), was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MI, USA). PC3, a cell line established from a grade 4 prostatic adenocarcinoma from a 62 year old male Caucasian (ECACC, #90112714, Lot No. 09K019, passage no. 9), was cultured in Coon's Modified Ham's medium supplemented with 10% fetal bovine serum, 2 mM glutamine 100 IU/ml penicillin and 100 μg/ml streptomycin. All cell lines supplied by ECACC underwent testing for mycoplasma by culture isolation, Hoechst DNA staining and PCR, together with culture testing for contaminant bacteria, yeast and fungi. Authentication procedures included species verification by DNA barcoding and identity verification by DNA profiling. Human cell lines were analyzed by PCR of short tandem repeat sequences within chromosomal microsatellite DNA (STR-PCR). Cell lines were initially expanded to form frozen stocks of cells and resuscitated cells were utilized for experiments up to passage 10.

In order to evaluate cell proliferation, the number of cells was measured by direct cell counting assay. Cells were plated at a density of 10^4 cells/cm^2, incubated in 5% CO_2 at 37 °C and recovered after different times of incubation. At the endpoint, cells were harvested, centrifuged and aliquots of cell suspensions were counted using a Neubauer hemocytometer chamber. Dead cells were assessed by the trypan blue dye exclusion test. For the hypoxia, cells were cultured in a Galaxy 48 R CO_2 incubator (New Brunswick Scientific, Enfield, CT, USA).

For immunofluorescence analysis, cells grown on coverslips (2×10^4 cells/cm^2) were fixed in 4% formaldehyde in PBS for 10 min at room temperature and permeabilized in PBS containing 0.1% Triton X-100 for 5 min. Cells were then incubated with primary antibody diluted in PBS containing 3% bovine serum albumin (BSA) for 1 h. After extensive washings with PBS, cells were treated with fluorescein-labeled IgG secondary antibody (1:100 in PBS containing 3% BSA) for 30 min at room temperature. After extensive washings, cells were mounted with Vectashield mounting medium and observed by fluorescence microscope equipped with a digital camera (AXIOPHOT, Carl Zeiss, Oberkochen, Germany).

Patients

A total of 35 prostate cancer patients were enrolled in our Urological Department (see data in Supplementary Tables S1–S3). The research has been carried out in accordance with the Declaration of Helsinki and approved by the Ethics Committee of our hospital. Written consent was obtained from all patients after full explanation of the procedure. The diagnosis of PCa was confirmed by the histopathological analysis of prostate biopsies with a final Gleason score assigned to every case. Pathologic stage categories were determined according to consolidated 1997 TNM staging system. Tumor volume was estimated after prostatectomy on the basis of measurement of the diameter of the largest tumor focus, as previously described [16]. The following anthropometric and clinical variables have been collected: body weight (kg), body height (cm), BMI calculated as body weight (kg) divided by body height (m) squared (kg/m^2), and waist-to-hip ratio (WHR). Systemic blood samples were drawn from overnight-fasting patients and used to measure testosterone and fasting insulin through routine from clinical laboratory analysis. Also fresh serum and plasma were obtained by blood samples for each patient and were stored at −80 °C until assayed. Serum leptin and adiponectin were measured by a quantitative sandwich enzyme-linked immunoassay (ELISA) technique using Quantikine Human Total Immunoassay (R&D Systems, Minneapolis, MN, USA).

Immunohistochemistry

Tissue samples were fixed in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2) and embedded in paraffin. Slide-mounted tissue sections (4-μm thick) were deparaffinized in xylene and serially hydrated in 100%, 95%, and 80% ethanol. Endogenous peroxidases were quenched in 3% H_2O_2 in phosphate-buffered saline (PBS) for 1 h and then slides were incubated with an anti-human primary antibody (10 μg/ml) for 1 h and then with peroxidase-conjugated secondary antibody for 30 min at room temperature. Sections were washed three times with PBS and antibody binding was revealed using the Sigma fast 3,30-diaminobenzidine tablet set (Sigma, St. Louis, MI, USA). Counterstaining was performed using hematoxylin solution. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The expression score was calculated by multiplying the number reflecting the dominant stain intensity (0, no detectable stain; 1, weak stain; 2, moderate stain; or 3, strong stain) by the number reflecting the percentage of positive tumor cells (0, no positive cells; 1, <10%; 2, 10–50%; 3, 51–80%; or 4, >80%). The resulting 12-point scale was categorized into three expression groups: 0 = no expression; 1–5 = weak expression; 6–12 = high expression.

Western blotting

Total cell lysates were obtained by incubating cells in a lysis buffer containing 1% triton, 0.1% SDS, 2 mM $CaCl_2$, 10 mg/ml orthovanadate, and 1× protease inhibitors cocktail (Sigma, St. Louis, MI, USA). Protein content was determined using the Protein Assay Kit 2 (Bio-Rad Laboratories, Hercules, CA, USA). Sixty micrograms of proteins was electrophoresed in 10% SDS–polyacrylamide gel. After electrophoresis, gels were placed onto Trans-Blot Turbo mini nitrocellulose transfer pack and transferred using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated with 1 μg/ml primary antibody and then with appropriate horseradish peroxidase-conjugated secondary antibodies. Primary antibodies MTCO1, β-actin, MCT-4, CD147, GLUT-1 and UCP2 were purchased from Santa Cruz Biotechnology; HIF-1α, SIRT1, cofilin, phospho-cofilin (Ser3), LIMK2, phospho-LIMK2 (Thr505) and secondary antibodies were purchased from Cell Signaling Technology; protein bands were visualized using a chemiluminescent detection system (Thermo Scientific, Rockford, IL, USA) and signals were digitally acquired by Chemidoc XRS system (Bio-Rad Laboratories).

Treatment with siRNA

In each well of a 6-well plate, 1×10^5 cells were plated in 2 ml of growth medium. When cells were ~50% confluent, they were transfected for 5 h at 37 °C with siRNA-SIRT1 or with scrambled sequence siRNA as control (final concentration 1 nM) using siRNA transfection Interferin reagent (Polyplus-transfection Inc, New York, NY, USA) and IBONI siRNA 4-duplexes (Riboxx Life Sciences, Radebeul, Germany). Cells were cultured with siRNAs for 24 h and then subjected to specific treatments for further 24 or 48 h.

Adhesion assay

Cells pretreated with leptin for 48 h were seeded at 5×10^5 cells/cm^2 in a 96-well plate coated with 10 μg/ml of extracellular matrix protein and incubated for 30 minutes at 37 °C in a 5% CO_2, 1% O_2, humidified atmosphere. Adherent cells were fixed with cold methanol for 10 min, washed with phosphate-buffered saline (PBS) and stained with 100 μl of 0.5% (w/v) crystal violet for 15 min at room temperature. Then cells were lysed with 2% (w/v) sodium dodecyl sulfate (SDS), 0.05% (w/v) sodium azide in water for 15 minutes with gentle agitation and solubilized stain was measured at 595 nm in a Bio-Rad Multiscan plate reader (Hercules, CA, USA).

Invasion assay

Cells pretreated with leptin for 48 h were trypsinized, washed twice with PBS, rinsed in complete medium and incubated at 37 °C for 30 min to reconstitute the membrane structures, washed again for FCS removal and then added to the upper compartment of collagen-coated or matrigel-coated transwell insert (Corning Inc, Tewksbury, MA, USA). Cells were allowed to migrate through coated filters for 8 h along a gradient of FCS. The cells attached on the lower membrane surfaces were stained with 0.1% (w/v) crystal violet in 0.1 M borate (pH 9.0) and 2% (v/v) ethanol for 20 min at room temperature. Cells were counted at ×400 magnification in standard optical microscopy and the average number of cells per field in 5 random fields