PGC-1α integrates glucose metabolism and angiogenesis in multiple myeloma cells by regulating VEGF and GLUT-4

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Abstract. Human peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) is a key coactivator in the regulation of gene transcriptional activity in normal tissues. However, it is not clear whether it is involved in the angiogenesis and metabolism of multiple myeloma (MM). The aim of the present study was to investigate the role of PGC-1α in MM. Small interfering RNA (siRNA) was used to inhibit PGC-1α expression in RPMI-8226 cells. An endothelial cell migration assay was performed using Transwell chambers and the expression of PGC-1α, estrogen-related receptor-α (ERR-α), vascular endothelial growth factor (VEGF) and glucose transporter-4 (GLUT-4) was tested by reverse transcription-polymerase chain reaction (RT-PCR). The protein expression of PGC-1α, ERR-α and GLUT-4 was assayed by western blot analysis. Lastly, RPMI-8226 cell proliferation was evaluated using CCK-8 assay. VEGF and GLUT-4 mRNA levels were decreased in cells treated with siRNA targeting PGC-1α, as was the level of GLUT-4 protein. Endothelial cell migration was significantly reduced when these cells were cultured with culture medium from RPMI-8226 cells treated with siPGC-1α. The proliferation rates at 24 and 48 h were suppressed by PGC-1α inhibition. Our results showed that inhibition of PGC-1α suppresses cell proliferation probably by downregulation of VEGF and GLUT-4. The present study suggests that PGC-1α integrates angiogenesis and glucose metabolism in myeloma through regulation of VEGF and GLUT-4.

Introduction

Multiple myeloma (MM) accounts for nearly 20% of mortality due to total hematologic tumors (1). The pathologic features of MM involve aberrant activation of metabolism and signal pathways, including uncontrolled and unlimited angiogenesis and increased glucose consumption. These changes play important roles in the clinical course of MM, particularly MM invasion and metastasis (2-4).

The angiogenic switch is controlled by the activation of pro-tumor genes such as hypoxia inducible factor-1 (HIF-1) and brain-derived neurotrophic factor (BDNF), which encode pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) (5-7). Studies have shown that angiogenesis is associated with upregulated HIF/VEGF pathways in ~40% MM patients (6), suggesting that other pathways could be involved in the regulation of VEGF besides the HIF/VEGF pathway.

PGC-1α is an important co-activator that participates in the regulation of gene transcriptional activity (8). It is now well established that PGC-1α contributes to several important functions, including mitochondrial biogenesis and metabolism, by interacting with transcription factors such as nuclear respiratory factors (NRFs) and estrogen-related receptor (ERR)-α (9-11). PGC-1α influences glucose consumption by regulating glucose transporter-4 (GLUT-4), which is upregulated in MM and is responsible for basal glucose consumption and maintenance of myeloid cell leukemia-1 (Mcl-1) expression, growth and survival (3). Studies have also found that PGC-1α promotes angiogenesis by binding with ERR-α, thereby increasing VEGF expression (12). Thus, angiogenesis and metabolism may be linked via PGC-1α. However it is not clear whether PGC-1α is involved in the regulation of angiogenesis and glucose metabolism in MM. Therefore, in the present study, we explored the role of PGC-1α in angiogenesis and glucose metabolism in MM.

Materials and methods

Cells and culture. Chemicals were purchased from HyClone, Sigma and Thermo Fisher Scientific, unless otherwise noted. The human MM cell line RPMI-8226 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and was grown in RPMI-1640 medium (HyClone, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin. Human umbilical vein endothelial cells (HUVECs) were cultured using the trypsin digestion method.

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All cells were grown at 37°C in an atmosphere containing 5% (v/v) CO₂. Peripheral blood mononuclear cells from normal healthy volunteers were separated and used as the normal control.

Cell proliferation assay with CCK-8 reagent. Cell proliferation was assayed using Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol (Dojindo Laboratories, Kumamoto, Japan). Cells were suspended at a concentration of 5x10⁴/ml in complete medium, seeded in 96-well plates at 0.1-ml suspension/well, and cultured at 37°C. Then, 10 µl CCK-8 solution was added to each well after 24 and 48 h of culture, respectively. After incubation at 37°C for 1 h, the plate was examined with a microplate reader (Bio-Rad, La Jolla, CA, USA) and the absorbance at 450 nm was recorded. Each experiment was performed in triplicate.

siRNA transfection. siRNA duplexes for ERR-α and PGC-1α were designed and produced by Shanghai GenePharma Co., Ltd. (Shanghai, China). RPMI-8226 cells were transfected with Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The sequence information for siRNA was: siERR-α, 5'-GGCAGAAAA CCAUUCUCAGGCUU-3' (sense) and 5'-CCUGAGAUG GUUUCUGCCUC-3' (antisense); siPGC-1α, 5'-GCGAAA CCAACAACUUUUU-3' (sense) and 5'-AUAAAGUU GUUGGUUUGGCUU-3' (antisense).

Migration assay. Endothelial cell migration was assessed as previously described (13). Briefly, 1.5-2.0x10⁴ of RPMI-8226 cells were loaded in the lower chamber of Transwell, and siRNA targeting PGC-1α or ERR-α was added. After 4-6 h of transfection, the culture medium was replaced with fresh RPMI-1640 medium, and then cultured for 24 h. Then, 1.5-2.0x10⁴ of HUVECs were seeded in the upper chamber (8-µm pore; Costar Corp., Cambridge, MA, USA). The plates were then cultured at 37°C in 5% CO₂ for 12 h. The cells migrated across the membrane and adhered to the lower part of the membrane, while those that did not migrate were removed with a cotton swab. The former were stained with crystal violet and examined under a microscope. The cell number before and after the experiments was counted in order to quantify proliferation.

RT-PCR. Total RNA was extracted using the TRIzol-based method (Sigma) from RPMI-8226 cells and the biopsy samples. Approximately 2 µg of total RNA was reverse-transcribed into the first-strand complementary DNA (cDNA) pool using a First-Strand cDNA Synthesis kit and real-time RT-PCR was carried out using SYBR-Green (Toyobo Co., Ltd., Osaka, Japan) with the Applied Biosystems 7500 System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Data analysis was carried out using the comparative Ct method. The following human-specific primers were used: β-actin, 5'-TTCCAAGCCTTCTCTCCTTG-3' (forward) and 5'-TTGCGTCTAGGAGCATA-3' (reverse); PGC-1α, 5'-TGGTGGCCACCACTCAAAGA-3' (forward) and 5'-TCACAAACAGGCAGACTG-3' (reverse); ERR-α, 5'-GTGG TATGAGGTGGTTGCT-3' (forward) and 5'-AGCGT CGGCACTTCCATGTG-3' (reverse); MEFC2, 5'-GCCCT GAGTCTGAGGACAAG-3' (forward) and 5'-AGTGAG CTGACAGGAGGTTGCT-3' (reverse); GLUT-4, 5'-GGCTTC TGTGACAGGGTTGCT-3' (forward) and 5'-CTCAGTT CTGTCGGTGTTTCC-3' (reverse); VEGF, 5'-AGTGAG GTGTCAGGATGTC-3' (forward) and 5'-GTGCTGGTTGCT-3' (reverse); PGC-1α, 5'-GGCAGAAAA CCAUUCUCAGGCUU-3' (sense) and 5'-CCUGAGAUG GUUUCUGCCUC-3' (antisense) and

GAGTCTGAGGACAAG-3' (forward) and 5'-AGTGAG CTGACAGGAGGTTGCT-3' (reverse); GLUT-4, 5'-GGCTTC TGTGACAGGGTTGCT-3' (forward) and 5'-CTCAGTT CTGTCGGTGTTTCC-3' (reverse); VEGF, 5'-AGTGAG GTGTCAGGATGTC-3' (forward) and 5'-GTGCTGGTTGCT-3' (reverse); PGC-1α, 5'-GGCAGAAAA CCAUUCUCAGGCUU-3' (sense) and 5'-CCUGAGAUG GUUUCUGCCUC-3' (antisense) and

Figure 1. Relative expression of PGC-1α and ERR-α in RPMI-8226 cells. mRNA of PGC-1α and ERR-α is present in RPMI-8226 cells and >7-fold higher than that of peripheral blood mononuclear cells from normal healthy volunteers. Levels are expressed as percentage relative to the values obtained for control cells.

Western blot analysis. Total cell proteins were prepared, fractionated and electroblotted on sodium dodecyl sulfate gels, and western blot analysis was performed as previously described (12).

Statistical analysis. To measure overall differences, particularly those between different treatments and control, SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used. Analysis of variance and post-hoc tests (two-sided Dunnett's t test) were applied to analyze the average values of replicate results obtained by independent experiments. A P-value of ≤0.05 was considered to indicate a statistically significant difference.

Results

PGC-1α is upregulated in MM. We found that the level of PGC-1α was upregulated in the MM RPMI-8226 cells. The level of ERR-α, which is a PGC-1α related co-activated factor, was also increased. The relative expression of PGC-1α and ERR-α in RPMI-8226 cells was >7-fold higher than that in the control (Fig 1).

PGC-1α regulates the expression of VEGF in vitro. The effect of PGC-1α and ERR-α on the expression of VEGF in the MM cell line RPMI-8226 was examined. PGC-1α was suppressed by siRNA in the RPMI-8226 cells, and the VEGF mRNA expression was then measured by RT-PCR. As shown in Fig. 2, our results showed that VEGF mRNA was significantly lower in the siPGC-1α group than in the control. Next, we treated the RPMI-8226 cells with siRNA targeting ERR-α and found that mRNA level of VEGF was also markedly reduced. These data indicated that both PGC-1α and ERR-α were required for the regulation of VEGF in RPMI-8226 cells, and these results are in accordance with those of other studies (12).
Suppression of PGC-1α inhibits in vitro angiogenesis in RPMI-8226 cells. In the present study, we also determined whether PGC-1α and ERR-α inhibition of MM cells affects the migration of human vascular endothelial cells. As shown in Fig. 3, RPMI-8226 cells without any treatment evidently promoted the migration of HUVECs, wherein a large number
of cells were observed to have crossed the membrane of the Transwell chamber. RPMI-8226 cells transfected with siRNA targeting PGC-1α or ERR-α inhibited HUVEC migration and the counts of the migratory HUVECs reduced.

Suppression of PGC-1α reduces GLUT-4 expression in MM. Myeloma cells exhibit upregulated expression of GLUT-4, which is necessary for glucose consumption, lactate production, growth and viability (3). We sought to determine whether PGC-1α plays a role in the regulation of GLUT-4 in MM. As shown in Fig. 4, suppression of PGC-1α led to decreased GLUT-4 expression in the RPMI-8226 cells.

Inhibition of PGC-1α results in growth and proliferation defects. Cells transfected with siPGC-1α or siERR-α were found to grow more slowly than the control cells. Similarly, the results of the CCK-8 assay also showed that proliferation of RPMI-8226 cells was diminished by siPGC-1α or siERR-α (Fig. 5), indicating that PGC-1α or ERR-α inhibition hampers the proliferation of myeloma cells.

Discussion

MM is a hematological malignancy characterized by the aberrant expression of malignant plasma cells within the bone marrow (14). A number of studies have shown that increased microvessel density (MVD) correlates with disease state, suggesting that increased bone marrow angiogenesis is important in myeloma progression (15). Other studies indicate that a high rate of glucose consumption beyond that necessary for ATP synthesis is exhibited by transformed cells including MM cells (16). This phenomenon has been further confirmed by F-18 fluorodeoxyglucose positron emission tomography-computed tomography (F-18-FDG PET-CT) scanning. Studies have shown that MM exhibits a high uptake rate of F-18 FDG and is positively correlated with the percentage of CD38/CD138-expressing myeloma cells in the bone marrow (17,18). Moreover, Kaira et al (19) showed that F-18 FDG uptake in cancers is determined by the presence of glucose metabolism, angiogenesis and other factors, suggesting that MM with high rates of F-18 FDG uptake is most likely characterized by increased angiogenesis and glucose metabolism.

Research on the formation of new blood vessels and VEGF in particular, is a major focus of MM investigations and has led to the clinical approval of monoclonal anti-VEGF agents (20). Although these agents show significant preclinical and clinical anticancer activity, they prolong overall survival of patients for months only, after which the tumor continues to grow. Therefore, an understanding of tumor angiogenesis is still needed (20).
The present study demonstrated that PGC-1α strongly regulated VEGF expression and angiogenesis by coactivating the orphan nuclear receptor ERR-α, suggesting that PGC-1α and ERR-α control a novel angiogenic pathway that delivers the needed oxygen and substrates (3). In addition, it has been suggested that activation of the ERR-α/PGC-1α pathway increases VEGF expression and angiogenesis (21), and that the ErbB2/Neu-induced mammary tumor cells ectopically expressing PGC-1α exhibit increased concentrations of the angiogenic factor VEGF compared with controls (22). Our results showed that PGC-1α and ERR-α are upregulated in MM and their expression was associated with the VEGF level. They also showed that PGC-1α or ERR-α inhibition can significantly suppress the expression of VEGF in vitro. Taken together, the results suggest that PGC-1α and ERR-α, major regulators of mitochondrial function and cellular energy metabolism, also play an important role in the regulation of VEGF and angiogenesis, not only in normal cells and tissues but also in MM. This also illustrates that only targeting the classical pathways involved in angiogenesis could probably not achieve the goal of anti-angiogenesis. Indeed, lenalidomide and other similar agents show limited efficacy in the treatment of MM, and this further supports the complexity of tumor angiogenesis. Targeting PGC-1α and/or ERR-α may improve the anti-angiogenesis effect of agents in clinical use.

Deregulation of glycolytic metabolism is another feature of MM (23). This observation and F-18-FDG PET results regarding MM suggest the reliance of myeloma on increased glucose consumption and glycolysis (24,25). Glucose is involved in generating energy-related chemicals, such as ATP, glucose also provides biosynthetic intermediates for lipid and nucleotide synthesis and plays a role in the regulation of several factors associated with cell death (i.e., Mcl-1, Bcl-2-associated death promoter protein) (29-32). The contribution of the glycolytic phenotype to increased resistance to apoptosis-inducing agents (33,34) supports the benefits of targeting glucose consumption.

Few studies have focused on determining the biological and molecular role of GLUT activation in tumors, knowledge that could facilitate the identification of potential therapeutic targets. McBrayer et al (3) performed gene expression profiling studies to identify deregulated GLUT family members in MM and demonstrated that myeloma cells exhibit reliance on constitutively cell surface-localized GLUT-4 for basal glucose consumption, maintenance of Mel-1 expression, growth and survival. It can, thus, be concluded that GLUT-4 is highly important to the MM proliferation and progression. GLUT-4 and its regulation controlled by other factors are well studied in normal cells. However, the pathway involved in regulating the expression of GLUT-4 is rarely studied in cancer, especially in MM. Our investigation of the interaction between MM cell proliferation and GLUT-4 activity delineates a pathway linking GLUT-4 activity with the aberrant expression of PGC-1α. PGC-1α inhibition can decrease GLUT-4 expression and results in hampered proliferation of RPMI-8226 cells. However, by silencing ERR-α expression, PGC-1α expression is as much as 7-fold increased and GLUT-4 expression is also increased, suggesting that ERR-α is a repressor of PGC-1α and that the latter is associated with GLUT-4 regulation, probably by enhancing transcription of the GLUT-4 gene (Fig. 6).

ERR-α is an orphan member of the nuclear receptor superfamily of transcription factors whose activity is regulated by the expression level and/or activity of its obligate co-regulator, PGC-1α. Under normal physiological conditions, and in response to different environmental stimuli, the ERR-α/PGC-1α complex is involved in regulating metabolic homeostasis under conditions of high energy demand in brown adipocytes, proliferating T cells and muscle. Notably, increased expression and activity of the ERR-α/PGC-1α axis have also been shown to correlate with unfavorable clinical outcomes in both breast and ovarian tumors (35). However, little is known about the role of ERR-α/PGC-1α in hematological malignances, particularly in MM.

In conclusion, our results demonstrate that PGC-1α and ERR-α are both upregulated in MM and human myeloma RPMI-8226 cells. Furthermore, this upregulation affects the in vitro expression of GLUT-4 and the angiogenesis in MM by increasing VEGF expression. Suppression of PGC-1α impairs the proliferation of RPMI-8226 cells, although not to a very great extent. Targeting PGC-1α may provide another effective and probably more potent way to curb the increased needs of glucose and angiogenesis in MM.

References


