Effect of EPA on Hsp 90 and GRα Protein Expression in Multiple Myeloma Drug-resistant Cells

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Abstract

Background

Approximately 20% of MM patients harbor glucocorticoid(GC) resistance and are not responsive to therapeutic effect. Chaperoneheat-shock proteins Hsp90 is needed for ligand docking, The imbalance of Hsp90/GRα(glucocorticoid receptor α) may be an important cause of GC resistance. Recent studies have indicated that EPA could repress cancer cell growth by regulating critical influential factors in progression of cancer, consisting of resistance to drugs, chemosensitivity. The aim of the present study was to test the cytotoxic effects of EPA alone or EPA+Dexamethasone in dexamethasone-resistant MM cell(MM.1R) and investigate whether DHA can induce apoptosis and reverse acquired glucocorticoid resistance in dexamethasone-resistant MM cell(MM.1R).

Methods

Cell Counting Kit-8 (CCK-8) was used to detect the proliferation of MM.1R cells after treating with EPA alone and EPA combined with DEX. Mitochondrial membrane potential was measured by flow cytometry and GRα and Hsp90 protein expression were assessed by western blot analysis.

Results

EPA alone was able to inhibit cell proliferation as evidenced by CCK-8 assay and the tumor growth was remarkably suppressed by EPA+Dexamethasone, Cell apoptosis after EPA treatment was obviously observed by Flow cytometry analysis of the mitochondrial membrane potential. Analysis of Hsp90 and GRα proteins in MM.1R cells incubated with EPA revealed down-regulation of Hsp90 and up-regulation of GRα. Accordingly, the Hsp90/GRα ratio was significantly decreased with the increase of EPA concentration.

Conclusions

EPA might be used as a new effective treatment for reversal of glucocorticoid-resistance in multiple myeloma.

Introduction

Multiple myeloma (MM) constitutes an hematological tumor of terminally differentiated bone marrow (BM) resident B lymphocytes referred to as plasma cells (PC)[1]. Despite improvements in biological knowledge, MM is still an incurable disease, due to the treatment-related mutations and drug resistance [2]. Therefore, new treatments strategies that either target the metastatic plasma cellsor the microenvironment of bone marrow can be considered in MM. Glucocorticoids(GC) is an important agent employed in all therapeutic formulations for treating leukemia, as well as multiple myeloma. The killing mechanisms of glucocorticoids include triggering of apoptosis of myeloma cells, decreasing thepoly (ADP ribose) polymerase cleavage as well as mitochondrial transmembrane potential and expression

level of caspase 3 [3]. Nevertheless, approximately 20% of patients harbor glucocorticoid resistance and are not responsive to therapeutic effect. Moreover, those that are responsive, may develop glucocorticoid resistance along the treatment time-course, resulting in a relapse, with a very dismal prognosis. Though some new findings provide new tools for overcoming glucocorticoid-resistance[4]. Enhancing glucocorticoid sensitivity is a growing challenge in the treatment of MM. Diverse mechanisms of resistance to glucocorticoids have been reported in MM. Functional abnormality of the glucocorticoid receptor underlies the occurrence of glucocorticoid resistance. A truncated glucocorticoid receptor(GR) without the C-terminal hormone docking domain has been reported in dexamethasone(DEX)-resistance MM cells[5]. There are two primary GR isoforms (α and β), with GRα isoform being the only GC functional receptor[6]. Studies suggested that GRβ has a prominent negative influence on GRα via GRα/GRβ heterodimer formation that prevents the GRα action[7, 8]. Unstimulated GRs remain in the cytoplasm as heterocomplexes with chaperoneheat-shock proteins, Hsp90, as well as Hsp70. Hsp90 is needed for ligand docking and Hsp70 is believed to facilitate ligand delivery to Hsp90 [9]. However, overexpression of Hsp90 has been documented to negatively modulate GR promoter activity, repress GR transcription, as well as diminish the presence of GR in the cytoplasm complex [10, 11].

Eicosapentaenoic acid (EPA), as well as docosahexaenoic acid (DHA) are long chain PUFAs. These PUFAs, as structural compartments of cellular membranes and as mediators of the intracellular metabolic cascades play pivotal functions in the cell. PUFAs have advantageous activitiesin diverse chronic conditions, such as neurodegenerative disorders, inflammatory, as well as cardiovascular diseases, and cancers[12–15]. Studies have indicated that EPA, as well as DHA repress cancer cell growth, as well as survival by regulating critical influential factors in progression of cancer, consisting of resistance to drugs, chemosensitivity, and angiogenesis[16–19]. The mechanism of cancer cell suppression by PUFAs includes induction of apoptosis[20, 21]. lipid peroxidation causing irreversible cell damage[22–24],and modulation of gene expression including that of transcription factors[25–28].

Previous study has shown that Hsp90 over expression has been reported in multiple malignant cell types including MM. To our knowledge, no previous studies exist concerning whether DHA can decrease Hsp90 expression in the GR complex and induce apoptosis, and reverse acquired glucocorticoid resistance in dexamethasone-resistant MM cell(MM.1R). Considering the anticancer capacity of DHA along with its limited side events on healthy cells, herein, we explored the effect of DHA on Hsp90, as well as GRα expression in multiple myeloma cells.

Materials And Methods

2.1. Reagents and antibodies

DEX-resistant human multiple myeloma cells (MM.1R) were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were preserved in our laboratory.Then they were taken out from the liquid nitrogen and characterized. Conventional resuscitation and subculture of MM.1R cells were performed. RPMI 1640 medium, as well as fetal bovine serum (FBS) were bought from Gibco (NY,

USA). EPA and DEX were purchased from Sigma; cell cycle detection kits and flow cytometry apoptosis detection kits were purchased from Nanjing KGI; anti-GR*α* antibodies (1: 1000), anti-Hsp90 antibody and GAPDH antibody were purchased from Abcam; protein lysate, BCA protein concentration determination kit, fluorescent secondary antibody, ECL chemiluminescence kit, CCK-8 kit were purchased from Shanghai Biyuntian Company.

2.2. Cell culture experiments

MM.1R cells were inoculated in DMEM enriched with fetal bovine serum (10%), streptomycin (100U/ml), as well as penicillin (100U/ml), and then incubated in an incubator programmed at 37°C and 5% CO_2. Growth media was refreshed every 2–3 days and passaging of the cells was performed accordingly, with the logarithmic phase cells selected for downstream experiments.

2.3. Group allocation

DEX was suspended in 0.05% DMSO, and dissolved to form a stock solution at a concentration of 10mmol/l, and then diluted at 1000 times with RPMI1640 medium. The final DEX concentration was adjusted to 10μmol /L. EPA reserve solution (100mmol/L) was prepared with anhydrous ethanol. The working solution(10, 20, 50, 100μmol/L) was then prepared by diluting the stock solution with RPMI1640. In the combined dosing group, different concentrations of EPA were incubated for 12h, An additional 10μmol/L DEX was introduced and incubation was continued for 24h, and then further experiments were performed. Equal volume medium was introduced to the control group. Growth of the control cells was conducted in medium with the similar concentration of DMSO or anhydrous ethanol as the DHA-possessing medium. EPA treatments at concentrations of 10–50μM suppressed cell proliferation remarkably.

2.4. Cell proliferation evaluation by the CCK-8 assay

Cells of logarithmic growth phase were collected. Digestion of the cells was performed using 0.25% trypsin, followed by cell suspension preparation. We adjusted the cell density to 1×10^5 cells/ml. Thereafter, the cells were planted in 96-well plate (200μl/well). The experiments were performed in 5 replicate holes. The cells were grown for 24h in an incubator programmed at 37°C, 5% CO2, as well as saturated humidity parameters. After adherent growth of the cells for 24h, the drug solution was added at various concentrations to the cells. CCK-8 reagent (10 μl) was introduced to each well, thorough mixing performed, then placed in an incubator for 2h. Absorbance values (OD value) were determined at 450nm using an enzyme standard instrument. The inhibition rate of cell growth= (test OD value − blank OD value)/ (control OD value − blank OD value) × 100%. The column charts of the final value were drawn and the result was the result analyzed afterwards. The experiment was replicated thrice and results averaged.

2.5. Flow cytometry analysis of the mitochondrial membrane potential

The mitochondrial membrane potential assay kit with JC-1 was employed to evaluate the mitochondrial membrane capacity. MM.1R cells in the log growth stage were planted (1×10^5 cells/well) in 6-well plates (2ml per well) and then different concentrations of drugs were added. Cells were collected through centrifugation 48 hours after the treatment. Samples were then rinsed with ice-cold PBS. Staining of the cells with 500μl JC-1 working solution was carried out at 37°C for 15min and the cells collected after sedimentation. After that, 500μl preheated JC-1 assay buffer was used to re-suspend the cells, and analyzed on a flow cytometer. The assay was replicated thrice and the average result used.

2.6. Western blot analysis of GR*α* and Hsp90 protein expression

The cells were processed as described above. MM.1R cells treatment methods ibid. After treating the cells for 24h, cells were collected, and the protein purification conducted using the RIPA lysate. Collection of the culture supernatantwas carried out, followed by centrifugation to remove any cell debris performed.Quantitation of the total protein was conducted using the BCA™ protein assay (Pierce). Fractionation of the total proteins was done using SDS-PAGE. After that, the proteins were transfer-embedded onto PVDF membranes. The blocking of the non-specific sites was performed via 1h incubation of the protein-embedded membranes with Tris-buffered saline-Tween20 (TBST) enriched with 3% bovine serum albumin at room temperature. Thereafter, incubation with primary antibody was conducted overnight in an incubator set at 4°C. Subsequently, the samples were rinsed thrice with TBST, and then conjugation with the secondary antibodies at a 1:5000 dilution for 2h was done via incubation at room temperature. After washing, color development of the protein bands was carried out using ECL for 3-5min. The bandintensity for each protein was scanned on a scanner and analyzed using the Imagaquent 5.1 image processing software. GAPDH was employed as a reference gene. The relative quantitative analysis was performed using the Imagaquent 5.1 software. The optical density of the target protein to GAPDH ratio was computed as the relative expression levelof the target protein in the samples. This experiment was performed thrice and results are presented as averaged values.

2.7. Statistical analyses

SPSS 16.0 software was applied in statistical analyses. All data were indicated as mean ± standard deviation (SD), the two-sample independent t test was used. $P < 0.05$ signified statistical significance.

Results

1. The influence of EPA on the proliferation of MM.1R cells

EPA treatments at concentrations of 10–50μM suppressed cell proliferation remarkably ($P < 0.05$ for all). In comparison to the single-drug treatments, the combination of EPA and DEX remarkably repressed cell growth. There was a statistically significance between the two groups at the same time point ($P < 0.05$). At the same time point, different concentrations of EPA(24□48h)on MM.1R cells The difference in the proliferation inhibition rate of MM.1R cells was statistically significant(Table 1).

Table 1
The influence of eicosapentaenoic acid (EPA) combined with dexamethasone (DEX) on the growth of MM.1R cells(%,x□±s)

GROUP	the inhibition rate of cell proliferation	
	24h	48h
control	0	0
10μmol /L EPA	8.45 ± 0.57[a]	12.07 ± 0.94%[ac]
20 μmol /L EPA	15.24 ± 1.27[a]	24.59 ± 1.29[ac]
50μmol /L EPA	21.97 ± 2.14[a]	35.47 ± 2.416[ac]
50μmol/L EPA + 10μmol /L DEX	38.78 ± 2.73[ab]	68.24 ± 2.02[abc]

Note: Compared with blank control group, [a]$P<0.05$; contrasted with 50μmol/L EPA group, [b]$P<0.05$; contrasted with 24h, [c]$P<0.05$. n = 3.

2. The influence of EPA on the mitochondrial membrane potential of MM.1R cells

One mechanism through which apoptosis occurs entails mitochondrial membrane integrity loss, as well as loss of transmembrane potential (i.e., collapse of Δψm). Changes in mitochondrial membrane potential, ΔΨm, are a pivotal step in cells undergoing apoptosis. We explored the impact of EPA on mitochondria membrane potential via fluorescent membrane-permeant JC-1 dye staining approach, as well as flow cytometric analysis. The JC-1 accumulation image indicates red fluorescence; the JC-1 monomer image indicates green fluorescence, the merged image combines the red and green images. Confocal microscopy demonstrated that EPA reduced the red JC-1 fluorescence and escalated green JC-1 fluorescence, respectively, leading to a reduction in the red/green fluorescence ratio. The fluorescence intensity gradually decreased with the progressive increase of EPA concentration and mitochondrial membrane potential in MM.1R cells drop to the maximum extent in the combined treatment group of EPA + Dexamethasone($P < 0.05$) (Fig. 1).

3. Western blot verification of the expression of Hsp90 and GR*α* protein

MM.1R cells were simultaneously treated with different concentrations of EPA for 24 hours. The Hsp90 content decreased gradually with the increase of EPA concentration and the expression of Hsp90 decreased significantly in the combined group of EPA + Dexamethasone. the GRα increased gradually with the increase of EPA concentration and when combined with Dexamethasone, the expression of Hsp90 is significantly increased. The effect appears to be dose dependent. Accordingly, the ratio of Hsp90 to GRα expression decreased gradually with the increase of EPA concentration ($P < 0.05$) (Table 2).

Table 2
The influence of of EPA on the Hsp90 and GRα protein expressions **(%,x̄±s)**

GROUP	Hsp90	GR*α*	Hsp90/GR*α*
control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
10μmol/L EPA	2.53 ± 0.05[a]	1.76 ± 0.03[a]	1.44 ± 0.05[a]
20μmol/L EPA	2.05 ± 0.03[a]	2.31 ± 0.04[a]	0.88 ± 0.02[a]
50μmol/L EPA	1.83 ± 0.05[a]	2.76 ± 0.04[a]	0.66 ± 0.02[a]
50μmol/L EPA + 10μmol/L DEX	1.58 ± 0.03[ab]	2.97 ± 0.08[ab]	0.53 ± 0.01[ab]

Note: Compared with blank control group, [a]$P<0.05$; contrasted with 50μmol/L EPA group, [b]$P<0.05$. n = 3.

Discussion

Herein, the efficacy of EPA in triggering cell apoptosis was explored in MM cells. We established that treatment of MM cells using different concentrations of EPA causes remarkable apoptotic death in a time-dependent, as well as dose-dependent approach. EPA reduced the membrane potential of mitochondria in MM cells, implying that apoptotic cell death of MM cells partly caspase-dependent. The proliferation assay showed that EPA exhibited a comparable inhibitory role with DEX in MM.1R cells. These data indicated PUFAs alone were insufficient to suppressU266 cell growth in vitro. According to theresults of MTT assays, EPA or DHA alone induced moderate U266 cell apoptosis but was insufficient to suppresscell growth without DEX treatment. These data demonstrated that PUFAs not only induced cell apoptosis butalso enhanced DEX sensitivity of U266 cells. The differences in metabolism, as well as structure between the malignant cells and the corresponding healthy cells account for the reported effect of EPA on malignant plasmacells, making the malignant cells more vulnerable to the EPA cytotoxic influence. EPA destroy the mitochondria to reduce mitochondrial membrane potential and ultimately induce apoptosis in MM .1R cells. Intriguingly, also the EPA cell apoptosis influences in acute myeloid leukemia cells were linked to mitochondrial metabolism disruption, stimulation of mitochondrial swelling,

as well asreduction of the potential of the mitochondrial membrane and was correlated with elevated oxidative stress, as well as dysregulation of Nrf2[29].

Chaperone Hsp90 has been documented as a pivotal factor participating in the GR complex stabilization, and only after dislocation of Hsp90, GR is translocated to the nucleus [30]. Molecular mechanism of GC unresponsiveness in some patients with multiple sclerosis might be related to increased presence of Hsp90 in the GR cytoplasmic complex, leading to the inhibition of GR translocation to nucleus and reduction in its transcription. The imbalance of Hsp90/GRα may be an important cause of GC resistance, an elevated Hsp90/GRα ratio affects the sensitivity to ligand at a step that is after receptor initiation [31]. Hence, we assessed Hsp90 linked to GRα in MM.1R. The Hsp90 increased gradually with the increase of EPA concentration. We additionally explored the ratio of Hsp90 to GRα expression and established that the ratio increased gradually with the increase of EPA concentration. These data suggest that EPA decreased the Hsp90 protein expression inthe GR complex and influences the GR function, leading to its enhanced responsivity to glucocorticoids.

In summary, our observations suggest that EPA might be used as a new effective treatment for reversal of glucocorticoid-resistance in multiple myeloma. The fact that EPA is active at physiological concentrations could necessitate further investigations of their clinical efficy in MM patients.

Declarations

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Availability of data and materials

All data and materials are available in this article.

Authors' contributions

Shenghao Wu participated in the design of the study, carried out the acquisition of data, participated in the manuscript drafting. Yuemiao Chen and Xueshuang Wang participated in the design of the study and the acquisition of data. Shanshan Weng was in charge of data gathering and computer programming for all data analysis. Wenjin Zhou participated in the design of the study. Zhen Liu conceived of the study,

participated in the design and coordination, drafted the manuscript. All authors read and approved the final manuscript. They all gave final approval of the manuscript and agreed to be accountable for its integrity.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Publication consent was obtained from all authors.

Competing interests

The authors declare that they have no competing interests.

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Figures

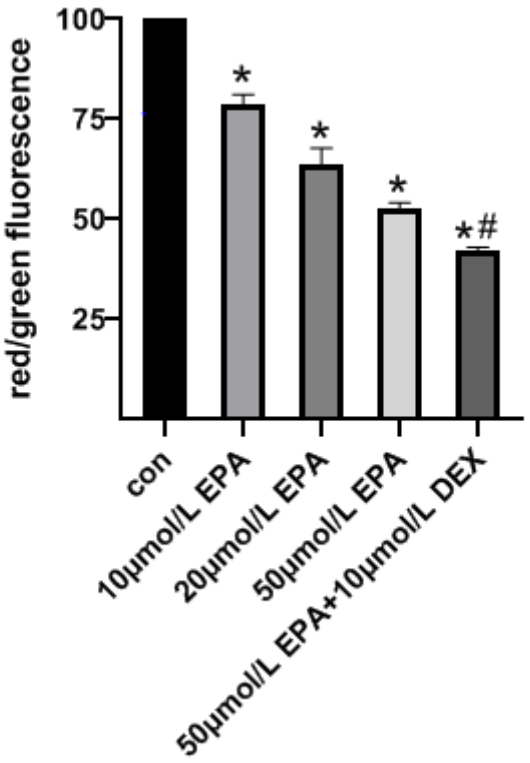

Figure 1

effect of EPA on apoptosis of MM.1R through the mitochondria pathway. Cells were exposed to indicated concentrations of 10μmmol/L EPA, 20μmol/L EPA, 50μmol/L EPA, 50μmol/L EPA+10μmol/L DEX for 48 h. Mitochondrial membrane potential was measured via JC-1 staining. Illustrative images of JC-1 derived fluorescence in MM.1R. Quantitative evaluation of the ratio of red/green fluorescence. *P<0.05 vs. control; #P<0.05 vs. 50μmol /L EPA, n=3.