Effects of Celastrol on growth inhibition of U937 leukemia cells through the regulation of the Notch1/NF-κB signaling pathway in vitro

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[Abstract] Background and Objective: Leukemia is a malignant tumor highly dependent on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is relevant for the occurrence, metastasis, proliferation, apoptosis, and drug resistance of tumor cells. Research has confirmed that the NF-κB family is one of the target genes in the Notch signaling pathway. This study investigated the effects of Celastrol on the apoptosis of U937 cells and the expression levels of Notch1 and NF-κB in these cells.

Methods: U937 cells were treated with various concentrations Celastrol (0.5–16.0) μmol/L for 12–60 h. MTT assay was performed to examine the effect of Celastrol on growth inhibition of U937 cells. Cell apoptosis was detected through both Annexin-V FITC/PI double-labeled cytochemistry and transmission electron microscopy (TEM). Cell cycle regulation was studied by propidium iodide. Western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) technologies were applied to assess the expression level of Notch1 in U937 cells. Subcellular distributions of NF-κB/p65 were detected through confocal microscopy.

Results: Celastrol presented striking growth inhibition and apoptosis induction potency on U937 cells in vitro in a time- and dose-dependent manner. The IC₅₀ value of Celastrol for 24 h was (6.21 ± 0.242) μmol/L. Moreover, Celastrol induced apoptosis in U937 cells in a cell-cycle dependent manner, which means that Celastrol could arrest U937 cells in the G₂/M phase. Through TEM, apoptotic bodies containing nuclear fragments were found in Celastrol-treated U937 cells. Overexpression of Notch1 was found in U937 cells, while Celastrol could downregulate it at both the protein and mRNA level in a dose-dependent manner, and expression of NF-κB decreased in nuclei and increased in the cytoplasm (P < 0.05). Conclusions: Celastrol inhibited cell proliferation and induced apoptosis in U937 cells in a concentration-dependent manner. The possible mechanism might be involved in the regulation of a survival signaling pathway, such as Notch or NF-κB.

Key words: Celastrol, U937 cells, Notch1, NF-κB, apoptosis
cancer cells. However, only sporadic reports about mechanisms of antitumor activity have been published in recent years. In this study, we used the Notch/NF-κB pathway as a molecular target to study the regulatory effects of different concentrations of Celastrol and the correlation its anti-leukemia effects.

Materials and Methods

Drugs and reagents
Celastrol, whose molecular formula is C29H38O4 with a molecular weight of 450 and a purity > 95%, was purchased from Sigma Aldrich (USA), dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. The RPMI-1640 medium and fetal calf serum was from Gibco (USA). Annexin-V/PI apoptosis kit was purchased from Shenzhen Jinmei Bio-Engineering Co. Ltd. Mouse anti-human NF-κB/p65 monoclonal antibody and rabbit anti-human Notch1 polyclonal antibody were purchased from the Santa Cruz Biotechnology, Inc. (USA). Fluorescein isothiocyanate (FITC) -marked goat anti-mouse IgG was purchased from Beijing Feiling Biotechnology, Inc. (USA). The reverse transcriptase polymerase chain reaction (RT-PCR) kit was purchased from Fermentas Life Sciences and primers were synthesized by the Shanghai Sangon Co., Ltd.

Cell lines and cell culture
Human myeloid leukemia U937 cells were purchased from the Cell Collection Center of Wuhan University. Cells were maintained in RPMI-1640 medium containing 10% inactivated fetal bovine serum, 100 u/mL penicillin, and 100 μg/mL streptomycin, and were kept in 5% CO2 at 37°C and saturated humidity. Cells were passaged with medium changes every 1–2 days. Cells in the logarithmic growth phase whose activity were above 98% were used in these experiments.

MTT assay for detecting the effects of Celastrol on proliferation
An amount of 1 × 10⁶/mL exponentially growing U937 cells were seeded into each well of 96-well plates and Celastrol was added at 0.25–16.0 μmol/L. Culture media with an equal volume of DMSO was used as a blank control. Three duplicates were created for each concentration with a total volume of 200 μL per well. Cells were incubated with 5% CO2 at 37°C for 12–60 h and then 20 μL of 5 mg/mL MTT was added into each well. After further incubation at 37°C for 4 h, the supernatant fluid was carefully removed and 150 μL of MTT was added to each well. Followed by shaking for 10 min to dissolve any remaining formazan, the optical density (A) at 492 nm was measured with a BioRad M450 microplate reader. Each experiment was repeated three times and the cellular proliferation inhibition rate (CPIR) was calculated using the following equation: CPIR = (1–mean A of experimental group/mean A of control group) × 100%.

Annexin-V/PI double staining for detecting apoptosis
Experiments were carried out according to manufacturer instructions. The samples were divided into experimental groups and a single-labeled control group. U937 cells treated with different concentrations of Celastrol and cells in the blank control group were collected. After rinsing twice with PBS precooled to 4°C, cells were resuspended in 100 μL of 1 x binding buffer at the density of 1 × 10⁶/mL. A volume of 5 μL of Annexin V-FITC and 10 μL of PI were added and mixed gently. The cells were incubated in the dark for 15 min at room temperature, then 300 μL of the buffers mentioned above were added. Machine detection was performed within 1 h.

Flow cytometry for detecting the cell cycle
A total of 1 × 10⁶ U937 cells in each treatment group were collected, washed in PBS twice, fixed with 70% cold ethanol overnight at 4°C, centrifuged, washed again in PBS once, and 20 μL RNase A were added, followed by incubating in a 37°C water bath for 30 min. Then 300–500 μL of propidium iodide (PI) dye was added, mixed, and incubated in the dark for 30 min at 4°C before flow cytometric analysis. The red fluorescence at an excitation wavelength of 488 nm was recorded.

Observation by transmission electron microscopy
Cells were collected in 1.5 mL of an EP tube. After centrifugation, the supernatant fluid was removed, and 2% glutaraldehyde was added, which were precooled to 4°C to fix for 2 h, then the cells were washed in PBS three times. Fixed in 1% osmium tetroxide for 1–2 h, gradient dehydrated with ethanol, saturated with 100% acetone epoxy, and embedded with epoxy resin, the cells were made into sections and dyed. Observations were performed with a transmission electron microscope (H-7500, Hitachi, Tokyo, Japan).

Semiquantitative RT-PCR assay for detecting the effects of Celastrol on the gene expression of Notch1
Total cellular RNA was extracted with a Trizol kit and complementary DNA (cDNA) was synthesized according to the instructions. Using the first strand cDNA of the cells as a template, PCR was performed. PCR primers were synthesized by the Shanghai Sangon Co., Ltd. The primer pairs of Notch1 were: 5’-CAGCGGCTGTACTCGGGCACAG-3’ (forward) and 5’-CAGAAGTGGTCGGAGAACTC-3’ (reverse), and the amplified fragment was 345 base pairs (bp). The primer pairs of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were: 5’-GGGACCTGACTAAcTACCTC-3’ (forward) and 5’-CAGTGATCTCCTTCTTCTC-3’ (reverse), and the amplified fragment was 623 bp. The amplification conditions of PCR were as follows: pre-denaturation for 2 min at 94°C, followed by a total of 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 58°C, and extension for 30 s at 72°C, then a final extension for 10 min at 72°C, after which the amplification was terminated. After identification by 1.5% agarose gel electrophoresis, the PCR products were photographed under ultraviolet (UV) light and scanning analysis was performed. Notch1/GAPDH were used in the semiquantitative analysis of gene expression levels.

Western blot analysis
The collected U937 cells treated with different concentrations of Celastrol and cells in the blank control group had 100 μL of precooled cell lysis buffer (prepared according to constructions of molecular cloning) added. After 30 min of lysis on ice, total cell
protein was extracted and quantified by Lowry assay. Gels and samples were prepared according to conventional methods for protein electrophoresis and the protein was transferred to the membranes. Rabbit anti-human Notch1 monoclonal antibody (1:1500) was added and the membrane was incubated at 4°C overnight. After rinsing, horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:2000) was added and the membrane was incubated on a shaker at 37°C for 1 h. Finally, electrochemical luminescence reagents were used in X-ray imaging and computer software was used to analyze the results. Each group at different concentrations was repeated three times and the mean values represented the results.

Confocal laser scanning microscopy for observing subcellular localization of NF-κB/p65

Two groups of cells were placed on the cell smear centrifuge. Slides were treated with 3-aminopropyltriethoxysilane (APES), then the cell smears were created. After fixation in 4% paraformaldehyde and perforation with 0.1% Tritro X-100, a working solution of goat serum was used to block for 1 h. Then 100 μL mouse anti-human NF-κB/p65 antibody (primary antibody) diluted in 1% bovine serum albumin (BSA) was added, followed by incubation at 37°C for 1 h. Then the cells were gently washed three times for 10 s each in PBS. As the negative control, 1% BSA instead of the primary antibody was used. A volume of 100 μL of FITC-conjugated goat anti-mouse IgG diluted in 1% BSA was added and the slices were incubated at 37°C for 30 min. After washing in PBS, slices were stored in a dark cassette. An inverted fluorescence microscope was used to observe the slices and a digital camera was used to take pictures. An image analysis system was applied to detect the gray value.

Statistical analysis

The experimental data was measured by x ± s. Comparisons of rates among the groups were performed using F-test. SPSS version 11.5 was used for statistical analysis.

Results

Effects of Celastrol on proliferation

As seen in Figure 1, after being treated with 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 μmol/L of Celastrol for 24–60 h, the proliferative activity of U937 cells in all groups was lower than in the blank control group. Celastrol below 1.0 μmol/L had little effect on proliferation. When the concentration of Celastrol reached 2.0 μmol/L, inhibition of proliferation increased significantly (P < 0.05). The inhibitory effects on proliferation increased significantly with the increase in exposure time and concentration of Celastrol in a time- and dose-dependent manner. The half maximal inhibitory concentration (IC50) of the group treated for 36 h was (2.031 ± 0.109) μmol/L.

Effects of Celastrol on apoptosis of U937 cells

As seen from TEM, the cell morphology of the control group was relatively normal, showing typical characteristics of tumor cells with few apoptotic cells. With the increase in concentration of Celastrol, more typical morphologic changes of apoptosis were observed in U937 cell, such as the microvilli on the cell surface disappeared, cell surface blebbing, cytoplasmic density increased, chromosomes condensed and marginalized, nuclei condensed, and apoptotic bodies formed (Figure 2). Subsequently, the Annexin-V/PI double-staining assay (Figure 3) quantitatively detected the effect of Celastrol on apoptosis of U937 cells. The results suggested that the proportion of apoptotic U937 cells gradually increased with the increase in the concentration of Celastrol. After being treated with 0.5, 1.0, and 2.0 μmol/L of Celastrol for 24 h, apoptotic rates were (9.97 ± 0.12)%, (18.02 ± 3.50)%, and (32.24 ± 3.92)%, respectively. There were significant differences compared with the (2.84 ± 1.88)% of the control group (P < 0.01).

Figure 1 Effects of Celastrol on the proliferation of U937 cells

Figure 2 Apoptotic morphologic changes of U937 cells induced by Celastrol for 24 h with transmission electron microscopy. Bar = 2.0 μm
Table 1 Effects of Celastrol on cell cycle distribution and early apoptosis (*n = 3, x ± s*)

<table>
<thead>
<tr>
<th>Celastrol (μmol/L)</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>Rate of Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>31.95 ± 1.84</td>
<td>46.45 ± 1.67</td>
<td>9.60 ± 1.23</td>
<td>2.78 ± 0.33</td>
</tr>
<tr>
<td>0.5</td>
<td>39.48 ± 2.30</td>
<td>42.19 ± 1.85</td>
<td>13.31 ± 1.84</td>
<td>2.69 ± 0.24</td>
</tr>
<tr>
<td>1.0</td>
<td>51.40 ± 1.91</td>
<td>36.67 ± 2.02</td>
<td>10.93 ± 0.98</td>
<td>4.25 ± 0.78</td>
</tr>
<tr>
<td>2.0</td>
<td>65.58 ± 2.83</td>
<td>22.29 ± 1.51</td>
<td>8.13 ± 1.02</td>
<td>12.77 ± 2.15</td>
</tr>
</tbody>
</table>

*P < 0.01 vs control group

Figure 3 Effects of Celastrol on cell apoptosis with Annexin-V FITC/PI assay. Cells were treated with various concentrations of Celastrol for 24 h.

A, control; B, Celastrol 0.5 μmol/L; C, Celastrol 1.0 μmol/L; D, Celastrol 2.0 μmol/L.

**Effects of Celastrol on the cell cycle of U937 cells**

After being treated with different concentrations of Celastrol for 24 h, the distribution of the cell cycle changed subsequently. The proportion of cells in the G0/G1 phase increased gradually with the increase in concentration of Celastrol, while cells in the S phase decreased in a concentration-dependent manner. The effect on cells in G2/M phase was not obvious (Table 1, Figure 4). The results suggested that Celastrol arrested the U937 cell cycle mainly at the G0/G1 phase.

**Effects of Celastrol on subcellular localization of U937 cells**

To better observe the subcellular localization of NF-κB/p65, we used laser scanning confocal microscopy to detect the distribution. After being treated with 2.0 μmol/L of Celastrol for 24 h, the fluorescence intensity of NF-κB/p65 decreased significantly and its subcellular localization also changed. NF-κB/p65 distributed in a circle around the cell surface, and the fluorescence intensity in cytoplasm decreased obviously (Figure 5).

**Effects of Celastrol on the gene regulation of Notch1 in U937 cells**

There was a high expression of the Notch1 protein in U937 cells. After being treated with 0.5–8.0 μmol/L of Celastrol for 24 h, the level of protein expression decreased significantly in a concentration manner (*P < 0.05*). To further clarify the effects of Celastrol on the regulation of the Notch1 protein, we detected the content changes of Notch1 mRNA at the level of gene transcription. Similarly, the expression level of Notch1 mRNA decreased in a concentration-dependent manner, which was obviously higher than that of the control group (Figure 6).

**Discussion**

Many natural agents, especially plants and food ingredients, have been found to have strong antitumor effects in vivo and in vitro. As early as the 1970s, tripterygium wilfordii was reported to have various effects, such as anti-inflammatory, analgesic, antioxidant, and antiviral effects, and to play a definite role in inducing tumor cell apoptosis. It was further identified that the characteristics of Celastrol, the active ingredient of tripterygium wilfordii, were different from those of general anticancer drugs. Celastrol could kill tumor cells selectively, while had no obvious damage to normal hematopoietic cells or the heart, liver, kidneys,
and so on. As a result, it was considered to be a safe and effective antitumor drug that can be continuously used in the long term. Some sporadic reports have been published about its antitumor mechanisms. This study searched for the possible upstream Notch1/NF-κB p65 signaling pathway, which has been associated with the apoptotic pathway, from a new perspective to further elucidate the effect of Celastrol on growth inhibition and the induction of apoptosis of U937 cells and to clarify its probable mechanisms.

Results showed that Celastrol can inhibit the proliferation of U937 cells and the inhibition was positively correlated with exposure time and concentrations. At the same time, Celastrol had a strong apoptosis-inducing effect. After being treated with 0.5~2.0 μmol/L Celastrol for 24 h, the apoptosis rate of U937 cells increased significantly and typical apoptotic morphologic changes were observed. The apoptosis-inducing effect of Celastrol may be closely related with its role in cell cycle arrest. Along with increasing concentrations of Celastrol, the proportion of cells in G0/G1 phase increased gradually and the proportion of cells in S phase correspondingly decreased gradually. However, Celastrol had little effect on proportion of cells in G2/M phase. Celastrol is speculated to induce apoptosis mainly through blocking U937 cells at the G0/G1 phase, coincident with this report.

In recent years, the Notch1 signaling pathway has been reported to be related to tumorigenesis. Most studies showed that the Notch signaling pathway plays a role in tumor promotion and is highly expressed in a variety of tumor cell lines and primary cells lines derived from diversified malignant tumors, such as hematologic malignancies, endometrial cancer, and colon cancer. The application of specific blockers of the Notch1 protein could inhibit proliferation and metastasis of the corresponding tumor cells and increase tumor sensitivity to chemotherapeutic drugs. The Notch1 protein is currently believed to exert its activity as an oncoprotein through protein-protein interactions with factors of tumor necrosis, integrin receptors, and vascular endothelial growth factor (VEGF). It was not hard to see that the Notch1 signaling pathway was a promising target in cancer therapy.

Therefore, we used the Notch1 gene as a target to observe the regulation of the traditional Chinese medicine preparation Celastrol on the Notch1 protein in U937 cells in this study. The results showed that U937 cells expressed high levels of the Notch1 protein. After treatment with Celastrol, the expression level of both the protein and gene decreased in concentration-dependent manners, which suggests that the effect of Celastrol on proliferation-inhibition of U937 cells was closely related to the Notch1 signaling pathway and further confirmed that the inhibition of the Notch1 signaling pathway could inhibit tumor cell proliferation and induce apoptosis. Celastrol is expected to become the next generation of Notch1-protein inhibitors.

NF-κB, which was first found in B cells in 1986, is a nuclear protein combined with enhancers of the immune globulin κ light chain. They are a group of transcription factors composed of Rel family members in homo- and heterodimerization. Generally, NF-κB combined with IκB is present in the cytoplasm in an inactive form in nonactivated cells. When externally stimulated, IκB phosphorylation occurs, followed by rapid degradation, and as a result, NF-κB is released and activated. Results in this experiment showed that, as a strong and effective apoptosis-inducing agent, Celastrol could regulate Notch1 at the gene and protein levels, as well as regulate the subcellular localization of transcription factors. After treatment with Celastrol, the fluorescence intensity of NF-κB in U937 nuclei gradually decreased and was expressed in the highest concentration on the membrane surface to retrieve normal functioning of nucleocytoplasmic shuttling. It suggested that hydrolysis of IκB decreased after treatment with Celastrol and it combined with NF-κB and suppressed NF-κB pathway, leading to reduced...
activity of NF-κB. When Celastrol induced apoptosis in U937 cells, expressions of Notch1 and NF-κB decreased in a concentration-dependent manner. In that way, what is the relationship between Noah-1 and NF-κB in the mechanisms of action of Celastrol for inducing apoptosis of leukemia cells Aifantis et al. [20] confirmed that NF-κB was a target gene of Noah-1, and that Notch1 downregulated the expression of NF-κB at the protein-protein and transcript levels through the recombinant signal-binding protein JK (RBP-JK). That gave rise to reduced activity of downstream NF-κB and eventual cell apoptosis. We speculated that the possible mechanism of Celastrol that induces apoptosis in U937 cells may be as follows: Celastrol acts on U937 cells→decreased expression of Notch1→reduced secretion of NF-κB→apoptosis of leukemia cells. Other studies had also confirmed that NF-κB is an important intermediate pathway in inducing tumor transforming and it may induce apoptosis of tumor cells through regulating the expression of NF-κB [21,22].

In sum, Celastrol significantly inhibited the proliferation of U937 cells and induced apoptosis. The effects of the Notch1 signaling pathway on the behaviors of tumors suggest that inhibiting its expression could inhibit the growth of tumor cells, promote differentiation or apoptosis of tumor cells, and reduce its invasive activity. This will lay a good foundation toward targeted therapy and drug screening.

References