Int J Clin Exp Med 2018;11(3):1699-1707
www.ijcem.com /ISSN:1940-5901/IJCEM0057946

Original Article

Prednisolone and chlormethine inhibit multiple myeloma through inhibition of Notch/NF-κB-mediated angiogenesis

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Received March 22, 2017; Accepted December 25, 2017; Epub March 15, 2018; Published March 30, 2018

Abstract: In multiple myeloma, bone marrow angiogenesis parallels tumor progression and correlates with disease activity. In this study, we examined the effects of prednisolone and chlormethine on myeloma cell viability, apoptosis and the expression of angiogenic factors. Human umbilical vein endothelial cells (HUVECs) and RPMI 8226 cells were treated with various concentrations of prednisolone and chlormethine at different time points. CCK-8 was used to analyze cell viability, and cell cycle and apoptosis were analyzed by flow cytometry. Real-time PCR and Western blot were used to measure the expression of angiogenic factors. Administration of prednisolone and chlormethine alone or combination inhibited cell viability, arrested cell cycle and induced cell apoptosis of HUVECs and RPMI 8226 cells. Treatment of HUVECs and RPMI 8226 cells with prednisolone and chlormethine alone or combination caused a decrease of angiogenic factors, such as Notch-1, NF-κBp65, VEGF, bFGF, MMP-2 and MMP-9. Prednisolone and chlormethine inhibit multiple myeloma angiogenesis by inhibiting Notch/NF-κB signaling pathway. Notch/NF-κB signaling pathway can be a target for the treatment of multiple myeloma.

Keywords: Multiple myeloma, prednisolone, chlormethine, angiogenesis, notch, NF-κB

Introduction

Multiple myeloma (MM) is still an incurable malignancy because of the development of drug-resistant phenotype after prolonged therapy [1, 2]. Combination chemotherapy offers initial response rates of 40% to 70% in MM patients, but refractoriness to these regimens eventually develops. High dose chemotherapy with stem cell support has achieved higher response rates than conventional chemotherapy [3], but few patients remain in long term remission.

Recent evidence underscores the possible role of angiogenesis in the progression of MM. It has been demonstrated that patients with active MM have increased bone marrow angiogenesis compared with those in remission. Moreover, it has been shown that microvessel density and angiogenesis in the bone marrow are correlated with the prognosis of MM and survival of patients [4, 5].

The molecular mechanism(s) by which Notch signaling induces tumor growth has not been fully elucidated. Notch-1 has been reported to cross-talk with another major cell growth and apoptotic regulatory pathway, namely NF-κB. NF-κB plays important roles in the control of cell growth, differentiation, apoptosis, and inflammation [6, 7]. Notch-1 has been reported to strongly induce NF-κB promoter activity in reporter assays [8] and to induce the expression of several NF-κB subunits [9]. cDNA enhanced NF-κB activity [10]. Down-regulation of Notch-1 caused attenuation of NF-κB consistent with the down-regulation of NF-κB downstream genes such as vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP-9), resulting in the inhibition of cancer cell invasion and angiogenesis [10].

Chlormethine, which chemically is the tertiary amine methylbisamine, is an alkylating agent that has been in use as part of combination regiments in treatment of Hodgkin's disease, non-Hodgkin's lymphoma, as palliative chemotherapy in lung and breast cancers and as a lotion to skin lesions of mycosis fungoides [11]. Similarly, because of its inhibitory activity on biochemical events associated with tumor development, prednisolone was also used alone or combined with other agents in treatment of MM [12], chronic obstructive pulmonary disease [13], acute herpes zoster [14], breast cancer [15], hormone refractory prostate cancer [16]. These suggest that prednisolone and chlormethine induce cancer cell death and tumor regression [17-19]. In this study, we examined the mechanism of antimyeloma effects of prednisolone and chlormethine on MM cells.

Materials and methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human cord veins as described previously and used in passages 3-6 [20]. Briefly, untraumatized umbilical cord segments, at least 20 cm in length, were cannulated and perfused with 200-400 ml phosphate buffered saline (PBS) to remove all traces of blood, then the vein lumen was filled with PBS containing 1 mg/ml collagenase I and incubated at 37°C for 10 min. The contents of the vein were gently flushed with an equal volume of PBS, centrifuged at 1000 rpm for 10 minutes and resuspended in M199 medium supplemented with 20% heat inactivated FBS, 50 ng/ml ECGS and 100 units/ml heparin. After 12 h, fresh medium was added. Thereafter, medium was exchanged at 48 h intervals. After 6 to 8 days, primary cultures formed uniform monolayers and 0.25% trypsin were used to harvest cells. RPMI 8226 cells were maintained in culture in RPMI-1640 supplemented with 10% heat inactivated FBS in an atmosphere of 5% CO_2 at 37°C.

Cell viability assay

The cell viability status was assessed by Cell Counting Kit-8 (CCK-8) (Beyotime, China). Briefly, HUVECs and RPMI 8226 cells were seeded in 96-well plates at the density of 2×10^3 cells/well with 100 ml culture medium. After cultured for 24 h, prednisolone (1, 5, 50 and 500 μmol/l), chlormethine (1, 5, 10 and 20 μg/ml) and combination of prednisolone (50 μmol/l) and chlormethine (5 μg/ml) were added to the medium respectively. Cells cultured in the medium without adding agents were taken as the control. The cells were then cultured for 0, 24, 48 and 72 h. Then, 20 ml of CCK-8 solution was added to each well, and the culture was incubated for another 3 h at 37°C. The optical density (OD) values were read at 450 nm by a microplate reader (Thermo, Waltham, MA, USA).

BrdU incorporation

After drug treatment for 48 h, the HUVECs and RPMI 8226 cells were harvested at a density of 1×10^5 cells/well and incubated with 10 μM BrdU for 60 min in the dark at 37°C. Then the cells were incubated with 1 ml of BrdU Staining Buffer for 2 h and washes with PBS prior to incubated with 5 μl of anti-BrdU for 1 h in the dark at room temperature. Data was acquired by flow cytometry (BD Biosciences, San Diego, CA, USA).

Cell cycle assay

Cell cycles were examined using PI and flow cytometry. The cells were seeded in 12-well plates at the density of 3×10^3 cells/well and then treatment with prednisolone (50 μmol/l), chlormethine (5 μg/ml) and combination of prednisolone (50 μmol/l) and chlormethine (5 μg/ml) for 48 h respectively. After treatments, the percentages of cells in the different phases of cell cycle were evaluated by determining the DNA content after propidium iodide (PI) staining. Briefly, cells were washed with PBS, trypsinized and centrifuged at 1,000×*g* at 4°C for 5 min. Pellets were fixed overnight in 70% cold ethanol. After fixation, cells were washed twice with PBS and incubated in PBS containing RNase (1 mg/ml) for 10 min at room temperature. Finally, samples were stained with PI (1 mg/ml) for 30 min at 4°C. Data acquisition was done by flow cytometry (EPICSXLMCL, Beckman Coulter, US) using Cell Quest software.

Apoptosis assay

Apoptosis was determined by flow cytometry analysis. The cells were collected after treatment with prednisolone (50 μmol/l), chlormethine (5 μg/ml) and combination of prednisolone (50 μmol/l) and chlormethine (5 μg/ml) for

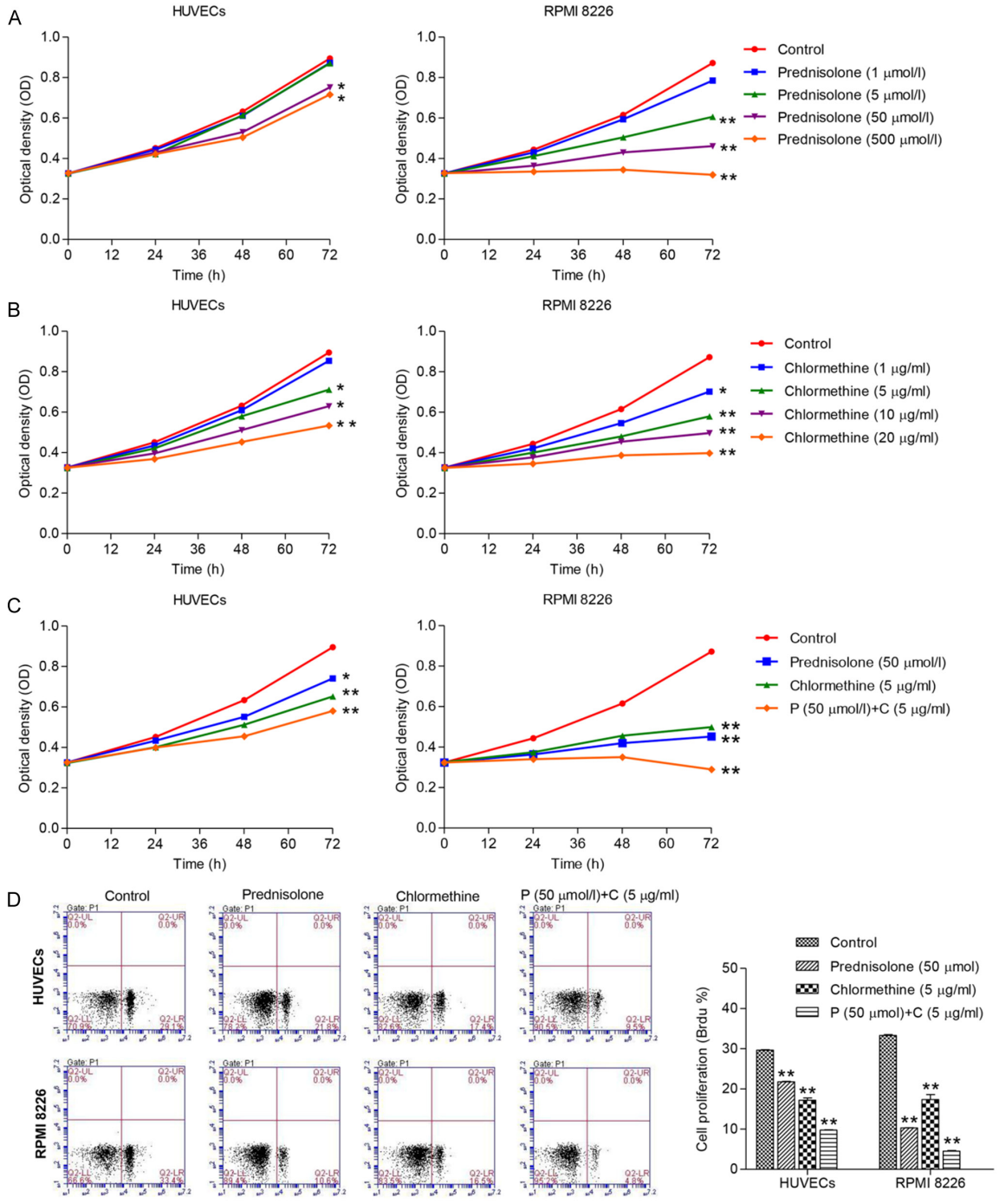

Figure 1. Prednisolone and chlormethine inhibit cell viability and proliferation of HUVECs and RPMI 8226 cells. HUVECs and RPMI 8226 cells were treated with different doses of prednisolone (1, 5, 50 and 500 μmol/l) (A), chlormethine (1, 5, 10 and 20 μg/ml) (B) and combination of prednisolone (50 μmol/l) and chlormethine (5 μg/ml) (C) for 0, 24, 48 and 72 h, CCK-8 was performed to identify the cell viability and the cell proliferation was measured by Brdu assay for 48 h (D). P: Prednisolone, (C) Chlormethine. Data were presented as mean ± SD, n=3. Symbols indicate 72 h were significant. **P*<0.05, ***P*<0.01 compared with control.

48 h respectively. Annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) double stain assays (BiovisionInc, Mountain View, CA) were performed following the manufacturer's protocol. Both floating and trypsinized adherent cells were all collected, resuspended in 500 μl of binding buffer containing 2.5 μl of annexin-V FITC and 5 μl of PI, and then incubated for 10

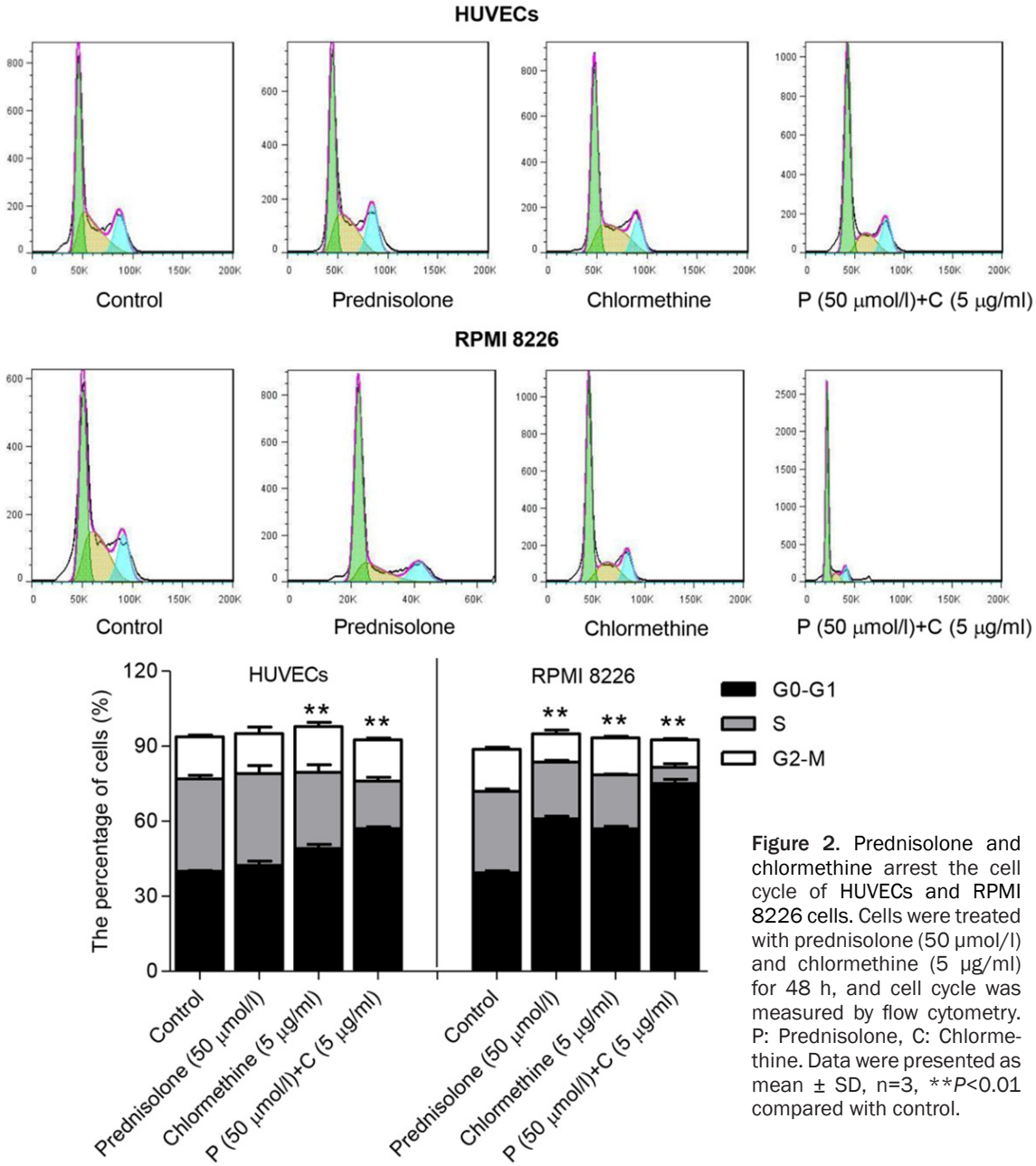

Figure 2. Prednisolone and chlormethine arrest the cell cycle of HUVECs and RPMI 8226 cells. Cells were treated with prednisolone (50 μmol/l) and chlormethine (5 μg/ml) for 48 h, and cell cycle was measured by flow cytometry. P: Prednisolone, C: Chlormethine. Data were presented as mean ± SD, n=3, $^{**}P<0.01$ compared with control.

min in the dark at room temperature before flow cytometry analysis.

Real-time RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Complementary DNA was synthesized with cDNA synthesis kit (Thermo Fisher Scientific, Rockford, IL, USA). Real time PCR was performed to detect mRNA levels of indicated genes. GAPDH was used an internal control. The gene expression was calculated using the ΔΔ Ct method. Primes were list as follows: Notch-1, 5'-GACGCACAAGGTGTCTTC-3' and 5'-TTGCCCAGGTCATCTACG-3'; NF-κBp65, 5'-GAATGGCTCGTCTGTAGTG-3' and 5'-TGGTATCTGTGCTCCTCTC-3'; GAPDH, 5'-CACCCACTCCTCCACCTTTG-3'.

Western blot

Following treatment with prednisolone and chlormethine at the desired concentrations, the cells were harvested. The expression of angiogenesis-related proteins was detected by Western blot according to the manufacturer's instructions and previous reports [21].

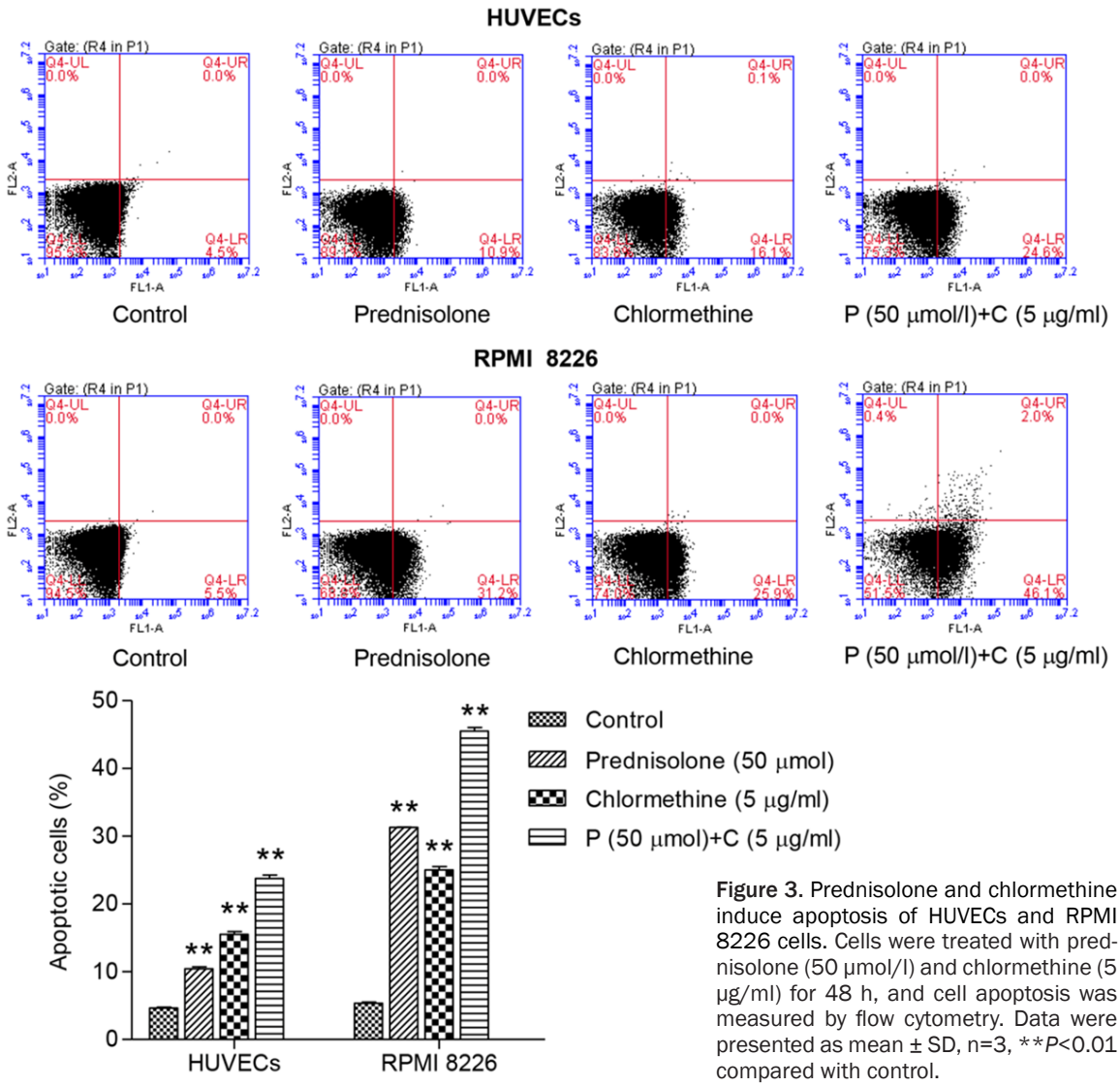

Figure 3. Prednisolone and chlormethine induce apoptosis of HUVECs and RPMI 8226 cells. Cells were treated with prednisolone (50 μmol/l) and chlormethine (5 μg/ml) for 48 h, and cell apoptosis was measured by flow cytometry. Data were presented as mean ± SD, n=3, $^{**}P<0.01$ compared with control.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was used as an internal control for whole cell lysates. The experiment was repeated three times independently.

Statistical analysis

The data was presented as the mean value ± SD. Data were analysed by one-way analysis of variance followed by Tukey's post hoc test. *P* value lower than 0.05 was considered to be statistically significant.

Results

Prednisolone and chlormethine inhibit cell viability of HUVECs and RPMI 8226 cells

We first examined whether prednisolone and chlormethine could inhibit cell viability of HUVECs and RPMI 8226 cells under alone and combination administration. As shown in **Figure 1A**, prednisolone had significant growth inhibition effects on the HUVECs and RPMI 8226 cells in a dose-and time-dependent manner ($^{*}P<0.05$, $^{**}P<0.01$). Cell viability was decreased remarkably after the cells were treated with prednisolone at 50 μmol/l for 48 h. Similarly, chlormethine also inhibited growth of the HUVECs and RPMI 8226 cells in a dose- and time-dependent manner and significantly decreased after the cells were treated with chlormethine at 5 μg/ml for 48 h (**Figure 1B**, $^{*}P<0.05$, $^{**}P<0.01$). It suggests that treatment of the HUVECs and RPMI 8226 cells with prednisolone and chlormethine results in a dose dependent inhibition of cell viability. Furthermore, we also investigated the effect of combination of these two agents on the cell

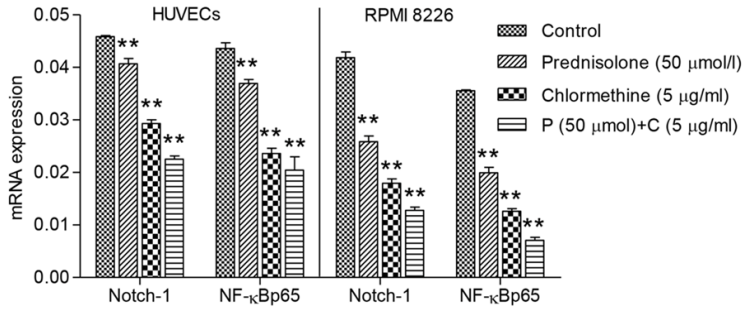

Figure 4. Prednisolone and chlormethine inhibit mRNA levels of Notch-1 and NF-κBp65 in HUVECs and RPMI 8226 cells. Cells were treated with prednisolone (50 μmol/l) and chlormethine (5 μg/ml) for 48 h, and the mRNA levels were measured by Real-time PCR. P: Prednisolone, C: Chlormethine. Data were presented as mean ± SD, n=3, $^{**}P<0.01$ compared with control.

viability, as shown in **Figure 1C**, combination of prednisolone and chlormethine was significantly inhibited cell viability compared with control and prednisolone or chlormethine alone administration ($^{*}P<0.05$, $^{**}P<0.01$). After drug treatment for 48 h, the cell proliferation was also measured by Brdu assay. As shown in **Figure 1D**, the cell proliferation was significantly decreased after prednisolone and chlormethine treatment in HUVECs and RPMI 8226 cells.

Prednisolone and chlormethine arrest the cell cycle of HUVECs and RPMI 8226 cells

We then examined the inhibition effect of prednisolone and chlormethine on cell cycle of HUVECs and RPMI 8226 cells. As shown **Figure 2**, the percentage of G0-G1 phase cells was increased especially in HUVECs treated with chlormethine alone and combined with prednisolone, respectively, from 49%±0.67% to 57%±2.8% (n=3) compared with control cells at 39%±1.2% ($^{**}P<0.01$). While the effect of prednisolone on cell cycle of HUVECs was not found. And the percentage of RPMI 8226 cells in G0-G1 phase was increased after prednisolone and chlormethine alone and combination treatment, respectively, from 61%±1.8% to 75%±2.9% (n=3) compared with control cells at 39%±1.3% ($^{**}P<0.01$).

Prednisolone and chlormethine induce apoptosis of HUVECs and RPMI 8226 cells

The effects of prednisolone and chlormethine on cell apoptosis were shown in **Figure 3**, administration of prednisolone and chlormethine alone and combination significantly increased the number of early apoptotic cells, respectively, from 10%±0.50% to 23%±0.85% (n=3) compared with control cells at 4.7%±0.21% in HUVECs ($^{**}P<0.01$), and significantly increased the number of early apoptotic cells, respectively, from 25%±0.90% to 45%±0.90% (n=3) compared with control cells at 5.3%±0.29% in RPMI 8226 cells ($^{**}P<0.01$). Similarly, the effect of combination of prednisolone and chlormethine was remarkably compared with other three groups.

Prednisolone and chlormethine inhibit mRNA levels of Notch-1 and NF-κBp65 in HUVECs and RPMI 8226 cells

To clarify the mechanism of antitumor effect of prednisolone and chlormethine, the Notch/NF-κB signaling pathway was detect by Real-time PCR. As shown in **Figure 4**, the mRNA levels of Notch-1 and NF-κBp65 were significantly decreased in HUVECs and RPMI 8226 cells, especially in combination of prednisolone and chlormethine compared with control and alone ($^{**}P<0.01$).

Prednisolone and chlormethine inhibit angiogenic factors expressions in HUVECs and RPMI 8226 cells

Figures 5 and **6** showed that the protein expression of Notch-1, NF-κBp65, VEGF, bFGF, MMP-2 and MMP-9 decreased in the cells administration of combined prednisolone and chlormethine compared with control and alone ($^{**}P<0.01$). However, the level of expression of GAPDH (as internal control) was not influenced by prednisolone and chlormethine.

Discussion

The interaction between myeloma cells and bone marrow microenvironment plays a crucial role in the pathogenesis of MM, and acquired drug resistance is also mediated by environmental factors. In particular, the role of abnormal, increased angiogenesis in the develop-

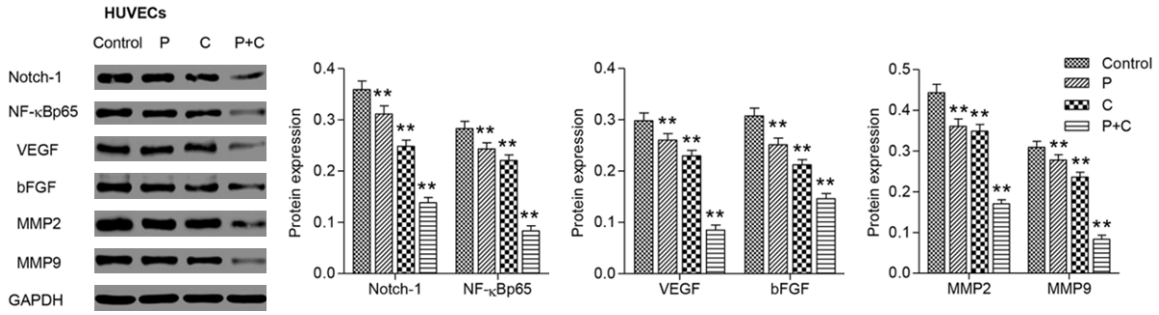

Figure 5. Prednisolone and chlormethine inhibit angiogenic factors expressions in HUVECs. Cells were treated with prednisolone (50 μmol/l) and chlormethine (5 μg/ml) for 48 h, and the protein levels were measured by Western blot. P: Prednisolone, C: Chlormethine. Data were presented as mean ± SD, n=3, $^{**}P<0.01$ compared with control.

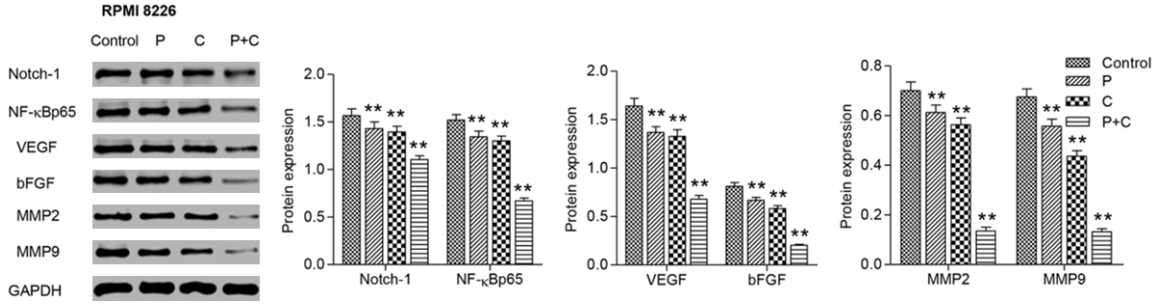

Figure 6. Prednisolone and chlormethine inhibit angiogenic factors expressions in RPMI 8226 cells. Cells were treated with prednisolone (50 μmol/l) and chlormethine (5 μg/ml) for 48 h, andthe protein levels were measured by Western blot. P: Prednisolone, C: Chlormethine. Data were presented as mean ± SD, n=3, $^{**}P<0.01$ compared with control.

ment and progression of MM has been well recognized [22]. MM cells are known to produce VEGF, bFGF, angiopoietin and other proangiogenic factors [23, 24]. New agents associated with antiangiogenesis activity such as thalidomide and VEGF receptor kinase inhibitor PTK787 have produced encouraging preliminary results [25, 26]. Therefore, in the present study we examined the inhibitory effect of prednisolone and chlormethine on myeloma cell dependent angiogenesis and the mechanism involved.

Prednisolone and chlormethine alone or combination treatment inhibited cell viability and proliferation of HUVECs and RPMI 8226 cells and the inhibition was concentration dependent. Moreover, prednisolone at 50 μmol and chlormethine at 5 μg/ml significantly inhibited cell viability and proliferation both in HUVECs and RPMI 8226 cells. Prednisolone and chlormethine also arrested cell cycle and induced cell apoptosis. Previous observations have shown that resveratrol, received wide attention because of its possible role in the prevention of human pathological processes, including cardiovascular diseases and cancer, is endowed with angiostatic activity in vitro and in vivo by inhibiting VEGF or bFGF triggered neovascularization in the mouse cornea and angiogenesis in tumor models [27, 28]. In order to examine whether prednisolone and chlormethine also have the anti-angiogenesis effect on MM, angiogenic factors were analyzed. According to our current work, prednisolone and chlormethine downregulated the expression of VEGF, bFGF, MMP-2 and MMP-9 in HUVECs and RPMI 8226 cells, which were the triggers of angiogenesis of MM. VEGF stimulates the proliferation, migration and tube formation of endothelial cells as well as the degradation of extracellular matrix (ECM), while bFGF plays an important role in proliferation, differentiation and survival of endothelial cells and may act together with other factors secreted by myeloma cells [29-31].

To investigate regulatory mechanisms, we examined the Notch/NF-κB signaling pathway by Real-time PCR and Western blot, we found

that the mRNA and protein levels of Notch-1 and NF-κBp65 were downregulated by prednisolone and chlormethine. Furthermore, down-regulation of Notch-1 caused attenuation of NF-κB consistent with the down-regulation of NF-κB downstream genes such as vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 and 9 (MMP-2, MMP-9), resulting in the inhibition of cancer angiogenesis. However, several groups have reported that NF-κB can also regulate Notch expression [32, 33]. The observations reported in the literature so far offer a complex and incomplete picture of the interactions between these two key cell fate-determining pathways. As is becoming increasingly clear in the case of other pathways, these interactions can be cooperative or antagonistic and multiple levels of feedback are possible depending on the context.

This study indicated that besides direct effects on MM cells, prednisolone and chlormethine exhibits antiangiognic effects. In conclusion, our study suggests prednisolone and chlormethine have strong antiangiogenic activity in MM cells, which its mechanism mainly through the inhibitory effects on the Notch/NF-κB signaling pathway and the expression of angiogenic factors.

Disclosure of conflict of interest

None.

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