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DUSP facilitates RPMI8226 myeloma cell aging and inhibited TLR4 expression

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Abstract. – OBJECTIVE: Myeloma severely threatens public health, and molecular targeting treatment becomes the future perspective. Dual specificity phosphatases (DSUP) protein has multiple functions including modulating cell proliferation, differentiation, aging, and apoptosis. Whether DUSP can regulate myeloma cell is unclear. This study thus aimed to investigate the effect of DUSP on myeloma cell line RPMI8226 cell aging and provide evidence for the clinical treatment of myeloma.

MATERIALS AND METHODS: H_2O_2-induced aging model of myeloma cell line RPMI8226 was generated. DUSP over-expression plasmid or specific siRNA was transfected by liposome. Western blot was used to detect the expression of DUSP in RPMI8226 cells. Cell aging condition was evaluated by β-galactosidase assay. Aging proteins P53 and P16 expression levels, the activation of TLR4 signal pathway were tested by immunoblotting. TLR4 signal pathway was then suppressed by Verteporfin for testing RPMI8226 cell aging.

RESULTS: Growing levels of DUSP, aging proteins P53 and P16, with inhibition of TLR4 signal pathway were found in the H_2O_2-induced aging model of myeloma cell line RPMI8226. Transfection of DUSP over-expression plasmid or siRNA potentiated or inhibited the aging of RPMI8226 cells induced by H_2O_2 and suppressed or enhanced TLR4 signal pathway, respectively. Verteporfin, an inhibitor of TLR4, increased the level of P53 and aging of RPMI8226 cells.

CONCLUSIONS: DUSP facilitates H_2O_2-induced aging of myeloma cell line RPMI8226 and suppresses TLR4 expression, which provides academic basis for clinical intervention.

Key Words:
DUSP, TLR4, Myeloma cells, Cell aging.

Introduction

Myeloma and other hematological diseases are major factors causing death in hematology cancers with prevalent incidence[1]. However, the mechanism of myeloma is not clear yet. A previous study[2] showed that enhanced growth/proliferation of myeloma cells, and decreased cell apoptosis/aging were major reasons for myeloma. Molecular targeting therapy represents the future direction for treating myeloma. Currently major treatment strategies for myeloma mainly target cell apoptosis, whilst few studies focus on cell aging[3,4].

Cell aging refers to a process of continuous weakening of cell growth, proliferation and differentiation potency with cell cycle progression being prolonged[5,6]. Growth and activity of cancer cells, however, are not impaired with normal cell activity, thus causing cell over-proliferation and tumorigenesis[7,8]. Therefore, the study of cell aging related mechanism is of critical importance for cancer prevention and treatment. Current study believed that cell aging is the result of deposited cell damage caused by oxygen free radicals after metabolism.

Current knowledge believes that Dual specificity phosphatases (DSUP) family member proteins play critical roles in cell aging[9,10]. DUSP is characterized with highly conserved amino acid sequence, with similar structures and functions, whilst different members may present unique functions[11,12]. DUSP family members contributed to a diversity of biological functions. For example, DUSP2 decreases cell proliferation velocity, whilst it is closely correlated with pulmonary carcinoma metastasis[13,14], thus indicating possible involvement of DUSP in myeloma occurrence and progression[15]. Therefore, this study used myeloma cell line RPMI8226 as the cell model, on which possible mechanism of DUSP on myeloma cell line RPMI8226 was investigated.

Materials and Methods

Myeloma Cell Model and Reagents

Myeloma cell line Roswell Park Memorial Institute (RPMI)8226 was purchased from ATCC

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(Manassas, VA, USA). Cell aging test reagent was bought from Dingguo Biotech (Beijing, China). Liposome transfection reagent was obtained from Invitrogen (Waltham, MA, USA). Antibiotics, cell culture medium, and fetal bovine serum (FBS) were provided from Beyotime (Beijing, China). Mouse anti-human DUSP, TLR4, P53, p16, and β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). siRNA-DUSP (5'-GGGAA ACAUU CAGAA ACUUtt-3' and 5'-AAGUU UCUGA AUGUU UCCCtg-3') and DUSP over-expression plasmid were purchased from Gimma Biotechnology (Shanghai, China).

Cell Culture and Generation of H_2O_2-Induced Myeloma RPMI8226 Cell Aging

Myeloma cell line RPMI8226 was resuscitated, precipitated and re-suspended in normal Dulbecco's Modified Eagle's medium (DMEM)[16]. Myeloma cell aging model was generated by adding 1 μl (10 μg/μl) H_2O_2 into RPMI8226 cells for further culture and assays.

Liposomal Transfection

As previously reported[17], liposomal transfection reagent was used to transfect siRNA DUSP and controlled siRNA and DUSP over-expression plasmid into myeloma cell line RPMI8226 as follows: cells were firstly cultured at 90% density. 1 μl (1 μg/μl) siRNA DUSP and controlled siRNA or DUSP over-expression plasmid were re-suspended into liposomal transfection reagent lipo2000. After 48 h incubation, culture medium was changed for further studies.

Cell Aging Assay

As previously described[18], liposomal reagent was used to transfect siRNA DUSP and controlled siRNA or DUSP over-expression plasmid into myeloma cell line RPMI8226. β-galactosidase assay was used to measure cell aging. Results were recorded under microscopy for analysis and statistics.

Western Blot for Protein Expression

After transfection with DUSP or siRNA-DUSP, myeloma RPMI8226 cells were collected and tested for cell lysate concentration. Western blot was performed starting with 60 V electrophoresis for 30 min, followed by 120 V electrophoresis for 120 min. After electrophoresis, proteins were transferred to NC membrane under 300 mA for 180 min. The membrane was then blocked with 5% defatted milk powder for 6 min at room temperature. Mouse anti-human DUSP, TLR4, P53, p16 and β-actin antibody (all diluted at 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, US) was added for 120 min at room temperature incubation. After PBST rinsing for three times, the membrane was developed under enhanced chemiluminescence (ECL) chromogenic substrate, followed by exposure and fixation in dark box. Imaging system was used for capture to analyze expression levels of DUSP and TLR4 among all groups of myeloma RPMI8226 cells (Bio-Rad, Hercules, CA, USA).

In measuring the effect of DUSP interference or overexpression on myeloma cell line RPMI8226, cells were collected after transfection and were tested as above-mentioned[15].

Statistical Analysis

All data were analyzed by SPSS 19.0 (SPSS Inc., Chicago, IL, USA) software and were presented as mean±standard error of means (SEM). The Student *t*-test was used for comparing among myeloma RPMI8226 cells. Continuous data are presented as means ± standard deviation (SD), and were analyzed by using one-way ANOVA, with the Tukey's post-hoc test. Significant level was defined when $p<0.05$.

Results

Rising Expression of DUSP and Suppressed TLR4 Signal in RPMI8226 Cell Aging Model

Elevating levels of aging proteins P53 and P16 were shown in myeloma cell line RPMI8226 after being induced by H_2O_2, indicating the establishment of successful aging model for further studies (Figure 1).

We further observed that in H_2O_2-induced myeloma cell line RPMI8226 aging model, DUSP level was apparently increased, suggesting the possible relation of DUSP to H_2O_2-induced myeloma cell aging. Also, the remarkable reduction of TLR4 was involved in myeloma cell aging model compared to normal control (Figure 1).

Transfection of DUSP Over-Expression Plasmid or siRNA Increased/Decreased DUSP Expression, Enhanced/Inhibited Cell Aging and Suppressed/Potentiated TLR4 Level in RPMI8226 Cells

To determine the effect of DUSP in cell aging, we over-expressed and down regulated DUSP. Of note, the increase of DUSP evidently elevated the expression of P53, while reduced the level of

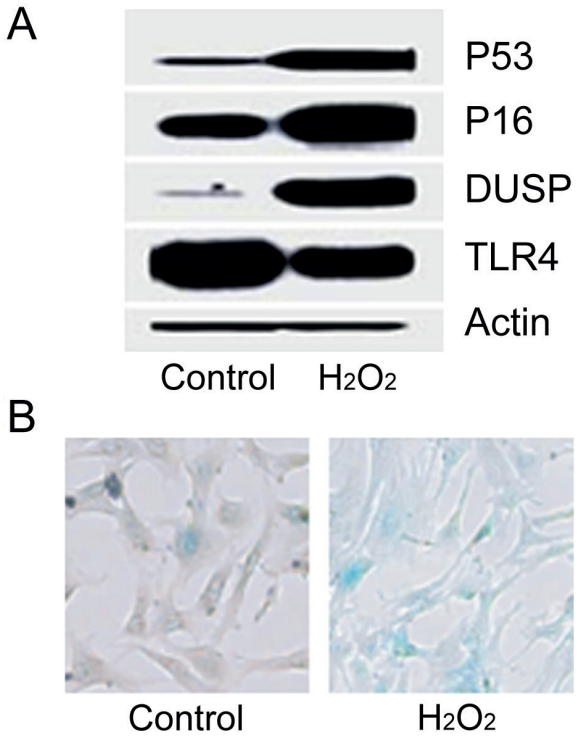

Figure 1. DUSP up-regulation and TLR4 signal pathway suppression in RPMI8226 cell aging model. ***A,*** Western blot test results. ***B,*** β-galactosidase staining for cell aging. Scale bar, 2 μm.

TLR4. However, the block of DUSP reversed the effect on P53 and TLR4, indicating that DUSP participated in H_2O_2-induced myeloma cell line RPMI8226 aging and might regulate the level of TLR4 (Figure 2)

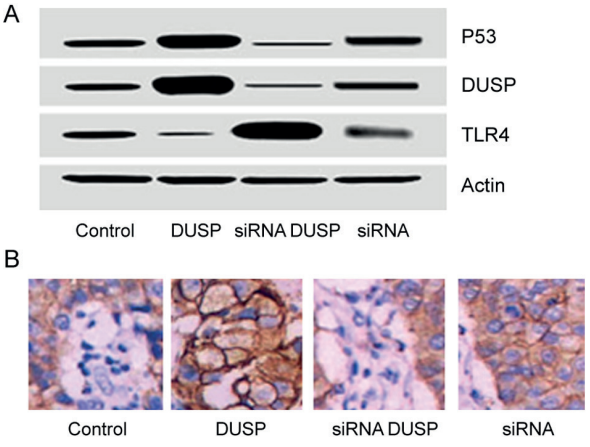

Figure 2. Transfection of DUSP over-expression plasmid or siRNA increased/decreased DUSP expression, enhanced/inhibited cell aging and suppressed/potentiated TLR4 signal pathway in RPMI8226 cells. ***A,*** Western blot results. ***B,*** β-galactosidase staining for cell aging.

Verteporfin Inhibited RPMI8226 Cell Aging

To further investigate the role of TLR4 in cell aging, Verteporfin, the inhibitor of TLR4 signaling pathway, was used to pre-treat cells to detect cell aging as a control. Interestingly, the TLR4 was inhibited by Verteporfin and, as result, the expression of P53 was markedly upregulated, while cell aging was induced by the detection of β-galactosidase assay. In a similar fashion, the growing level of DUSP also presents the same effect on P53 expression and cell aging, in addition to inhibition of TLR4 as Verteporfin did, indicating that DUSP favored H_2O_2-induced myeloma cell line RPMI8226 aging via suppression of TLR4 (Figure 3).

Discussion

In our study, we established myeloma cell line RPMI8226 by H_2O_2 induction as an *in vitro* model to investigate regulatory role of DUSP on tumor cell aging. Data showed that transfection of DUSP enhanced expression level of aging molecules in human myeloma cell line RPMI8226, as consistent with previous reports[19,20] showing the participation of DUSP in cell growth and aging. It was shown that DUSP2 down-regulation suppressed tumor cell

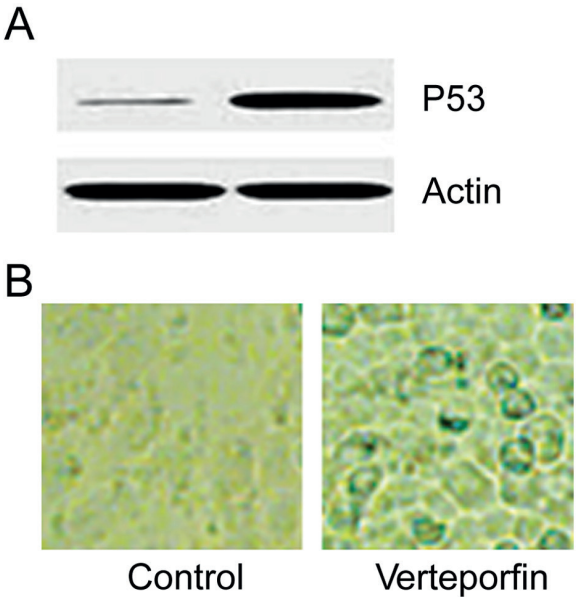

Figure 3. TLR4 signal pathway inhibitor Verteporfin significantly enhanced H_2O_2-induced myeloma cell line RPMI8226 aging. ***A,*** Western blot test results. ***B,*** β-galactosidase staining for cell aging. Scale bar, 2 μm.

proliferation, whilst DUSP1 was proposed to be correlated with tumor metastasis[21,22]. These results indicated that DUSP might be involved in myeloma occurrence and progression[23-25].

Selman et al[26] also indicated TLR4 protein as one inhibitory protein for cell aging. Current investigation believed that certain endogenous TLR-recognized ligands were produced during occurrence and progression of myeloma, indicating potential roles of TLR in tumor immune surveillance[27]. Intriguingly, results from this work showed that transfection of DUSP down regulated TLR4 level, and increased aging rate of myeloma cell line RPMI8226. The application of TLR4 signal pathway inhibitor, Verteporfin, enhanced RPMI8226 cell aging. DUSP affected myeloma cell growth. TLR4 is one widely recognized cell aging inhibitor. However, whether DUSP can modulate RPMI8226 cell growth or aging via TLR4 signal pathway is still unclear[27,28]. Accumulative evidence showed that various factors, such as USP22, sirtuin 4, contributed to the regulation of cancer cell aging via distinct signaling pathways[29,30]. Our data provided possible correlation of DUSP and TLR4 on the regulation of cell aging. The limitation of our research is that extensive types of tumor cells as well as *in vivo* assay need to be used to validate the DUSP and TLR4 on cell aging. Moreover, the downstream targets of DUSP, such as TLR4, or other alternatives, require further investigation.

Conclusions

We showed that DUSP could promote H_2O_2-induced myeloma cell line RPMI8226 aging and inhibited TLR4, which plays as a suppressor of cell aging. Our finding provides novel strategy for anti-myeloma, indicating that TLR4 might be the possible target for treating myeloma.

Acknowledgments

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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