Int J Clin Exp Med 2019;12(12):13586-13591
www.ijcem.com /ISSN:1940-5901/IJCEM0064527

Original Article

Molecular mechanism of NF-κB signal pathway in autophagy of retinoblastoma cell line HXO-RB44

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Received August 28, 2017; Accepted March 12, 2018; Epub December 15, 2019; Published December 30, 2019

Abstract: The pathogenic mechanism of retinoblastoma (RB) is still unclear. Abnormal cell autophagy is closely correlated with eye disease especially RB. This study thus investigated the role of cell autophagy disorder in the pathogenesis of RB and clinical implication. Using RB cell line HXO-RB44 cell as the model, UV irradiation was performed. Western blot measured autophagy condition of cells, as demonstrated by the expression level of related molecules and activation of signal pathways such as NF-κB. Autophagy inhibitor was used to treat HXO-RB44 cells, whose expression level of autophagy-related molecules and activation of NF-κB pathway were measured. NF-κB activator or inhibitor was further used for treatment, followed by measuring autophagy and NF-κB activation. The correlation between NF-κB signal pathway and RB pathogenesis was investigated. UV irradiation of RB cell line HXO-RB44 led to cell autophagy and NF-κB signal pathway activation. Inhibition of NF-κB pathway suppressed UV-induced autophagy of HXO-RB44 cells, whilst over-expression of NF-κB signal pathway enhanced UV-induced cell autophagy. The condition of NF-κB activation in RB tissues and their autophagy levels were closely correlated with disease severity. UV induces RB cell autophagy possibly via NF-κB signal pathway, suggesting the management of RB cell autophagy might be one strategy for treating RB.

Keywords: UV irradiation, NF-κB signal pathway, retinoblastoma cells, cell autophagy

Introduction

Retinoblastoma (RB) has high incidence and severely affects patients' physiological function and mental health [1]. Therefore, it is of critical importance to investigate the pathogenic mechanism of RB, which is still unclear, however. Multiple factors such as chemical reagents benzopyrene, viral carcinogen, chronic ulcer, ionization irradiation, inflammation and high dosage of UV irradiation all may lead to RB occurrence [2-4].

Clinical strategy for RB treatment is early diagnosis and treatment. Although classical treatments including radiation, chemotherapy and surgery have obtained satisfactory efficiency, chemo- or radio-therapy frequently lead to internal organ hemorrhage, immune suppression and dazzle or other adverse conditions [5]. The improvement of RB treatment accuracy and successful rate is thus one major challenge in medical science. Precise treatment is the ultimate solution for RB [6, 7], although the choice of targets is the major challenge. Therefore, it is urgent to discover more effective molecular targets for RB treatment. More importantly, the treatment strategy for RB targeting NF-κB signal pathway is still lack [8, 9].

NF-κB signal pathway has a wide array of functions, including inhibition of RB cell growth, and its participation in the metastasis process of liver cancer or pulmonary carcinoma [10]. All these studies indicate possible involvement of NF-κB signal pathway in RB pathogenesis and progression [11]. This study thus used RB cell line HXO-RB44 as the experimental model, on which possible regulatory role of UV on HXO-RB44 cells was investigated.

Cell autophagy is an auto-regulatory process of cells via a series of autophagy related proteins and signal pathways [12, 13]. In addition, autophagy participates in aging related disease occurrence and NF-κB signal pathway is widely studied regarding its role in facilitating autophagy [14-16]. Currently few drugs have been developed targeting NF-κB signal pathway proteins, plus unsatisfactory efficiency of NF-κB

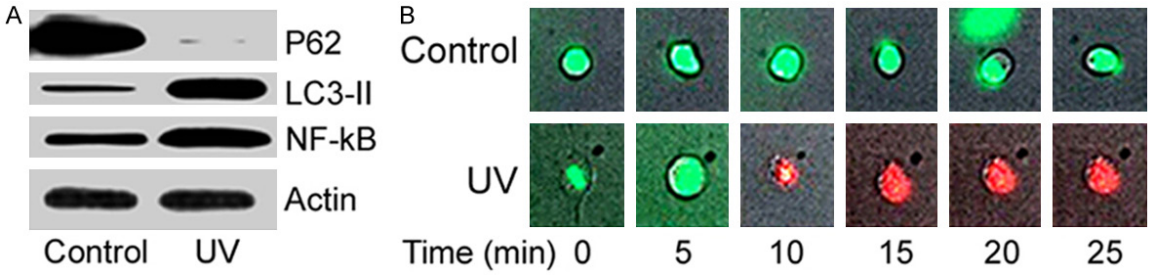

Figure 1. UV irradiation led to autophagy and NF-κB activation of HXO-RB44 cells. A. Western blot for cell autophagy; B. Confocal microscopy for cell autophagy, blue for P62 staining of nucleus, green for LC3 staining representing cell autophagy.

signal pathway protein suppression [17, 18]. This study thus aimed to investigate targets for suppressing NF-κB signal pathway.

Using RB cell line HXO-RB44 as the cell model, this study discussed possible regulatory function of UV on RB cells and related mechanisms, in order to provide evidences for developing treatment target of RB.

Reagent and method

Cell model and reagent

RB cell line HXO-RB44 was purchased from American Microbial Conservation Center (US). Assay kits for cell autophagy were purchased from Beyotime (China). Fetal bovine serum (FBS) and DMEM culture medium were obtained from Hualan Bio (China). Other reagents were purchased from Santa Cruz (US).

Cell culture

RB tumor cell line HXO-RB44 was resuscitated and re-suspended in high-glucose DMEM culture medium for incubation [19].

Cellular NF-κB signal pathway activity assay

NF-κB signal pathway activity assay kit was used to test HXO-RB44 cell activity following routine methods [20]. In brief, cells were cultured and treated by UV irradiation as described below. 2 mg/ml cell activity assay buffer was added into each well for 4 h culture, followed by adding DMSO to quench the reaction in 5 min. After quenching, HXO-RB44 cells incubated in 24-well plate was loaded into the microplate reader for measuring absorbance values at 560nm wavelength to plot a growth curve of RB cell line HXO-RB44 [21].

Western blot assay for cell autophagy

HXO-RB44 cells after UV irradiation or NF-κB signal pathway inhibitor/activator treatment were collected and prepared for total protein suspensions following the manual instruction. Protein concentration was quantified by BCA test kit. Cell lysate was extracted and quantified by microplate reader. Proteins were separated by centrifugation. Equal volume of cellular protein suspension (containing 20 μg proteins) was boiled for 10 min, followed by Western blot using 12% separation gel. Electrophoresis was performed at 60 V for 30 min firstly, followed by 120 V for 90 min. After that the membrane was transferred at 90 mA for 180 min. The membrane after transferring was blocked in 5% defatted milk powder for 60 min at room temperature, followed by primary antibody (ant-NF-κB at 1:1000 dilution) incubation at 4°C overnight. After rinsing in TBST to remove primary antibody, secondary antibody with horseradish peroxidase conjugation (anti-mouse IgG at 1:2500) was added for 37°C incubation for 3 h. The membrane was rinsed in TBST three times and was developed by ECL chromogenic substrate buffer. Gel imaging system (Qinxiang, China) was used to analyze gray intensity of all protein bands, in order to compare expression level of NF-κB signal pathway proteins in HXO-RB44 cells from all treatment groups [22].

Immunofluorescence for cell autophagy

UV or NF-κB signal pathway inhibitor/activator treated HXO-RB44 cells were tested for autophagy level using immunofluorescence method.

Statistical analysis

SPSS 15.0 was used for data analysis. Between-group comparison was performed by

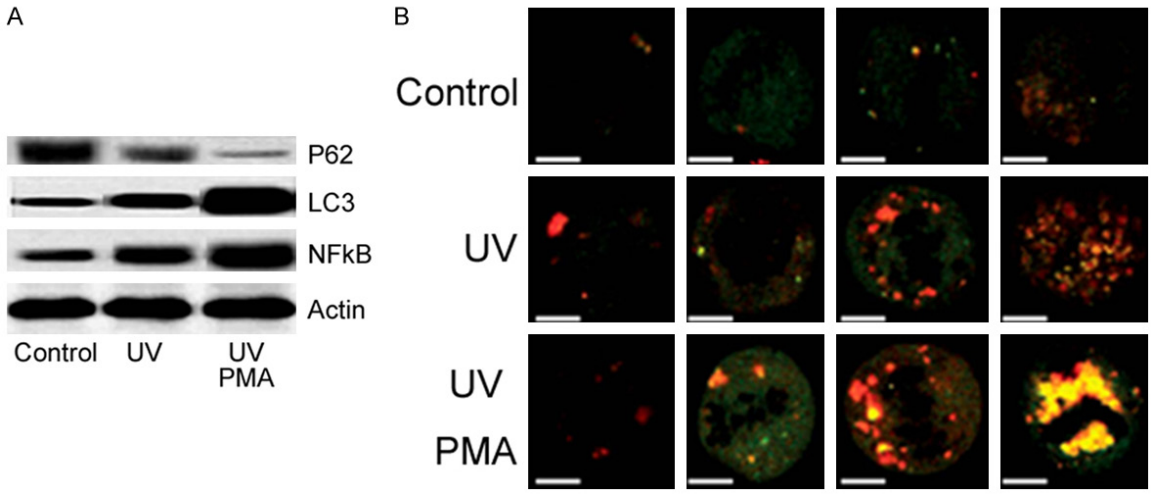

Figure 2. NF-κB signal pathway activator enhanced UV-induced autophagy of RB cell line HXO-RB44. A. Western blot for cell autophagy; B. Confocal microscopy for cell autophagy, blue for P62 staining of nucleus, green for LC3 staining representing cell autophagy.

student t-test among all groups of HXO-RB44 cells. A statistical significance was defined when $p<0.05$.

Results

UV irradiation led to autophagy and NF-κB activation of HXO-RB44 cells

As shown in **Figure 1**, UV irradiation on HXO-RB44 cells led to activation of NF-κB signal pathway, as control and UV groups showed 1.0 and 5.2 ± 0.7 fold increase of NF-κB relative activity demonstrated by Western blot analysis. UV irradiation also caused increased cell autophagy, as immunofluorescence showed 0 and 83.4% ± 5.8% of autophagy level.

NF-κB signal pathway activator PMA enhanced UV-induced autophagy of RB cell line HXO-RB44

As shown in **Figure 2**, treatment of HXO-RB44 cells using NF-κB signal pathway activator PMA enhanced the occurrence rate of UV-induced cell autophagy by 57%.

NF-κB signal pathway inhibitor suppressed UV-induced autophagy of RB cell line HXO-RB44

As shown in **Figure 3**, treatment of HXO-RB44 cells using NF-κB signal pathway inhibitor BAY11-7082 suppressed the occurrence of UV-induced cell autophagy.

Correlation analysis between NF-κB signal pathway activity and autophagy level

As shown in **Figure 4**, NF-κB signal pathway activation condition was positively correlated with cell autophagy level (correlation coefficient R=79.5%, p=0.0052).

Discussion

RB severely threatens patient daily life and health. This study utilized RB cell line HXO-RB44 as the cell model, on which regulatory role of UV on RB cells and possible mechanisms were investigated. Results showed that UV potentiated autophagy of HXO-RB44 cell, as consistent with previous study showing the participation of UV in cell autophagy [23]. Abnormal cell autophagy is correlated with various diseases including aging, cardiovascular disease, neurodegenerative disease and RB [4]. However, the signal transduction pathway of autophagy in RB pathogenesis is still unclear. This study investigated the role of NF-κB induced cell autophagy in RB pathogenesis, in addition to its potential clinical implications.

Clinical strategy for RB is early and timely diagnosis and treatment. Although classical treatments such as radiotherapy has obtained satisfactory efficiency, chemo- or radio-therapy frequently lead to internal organ hemorrhage, immune suppression and dazzle or other adverse conditions [5]. The improvement of RB treatment accuracy and successful rate is th-

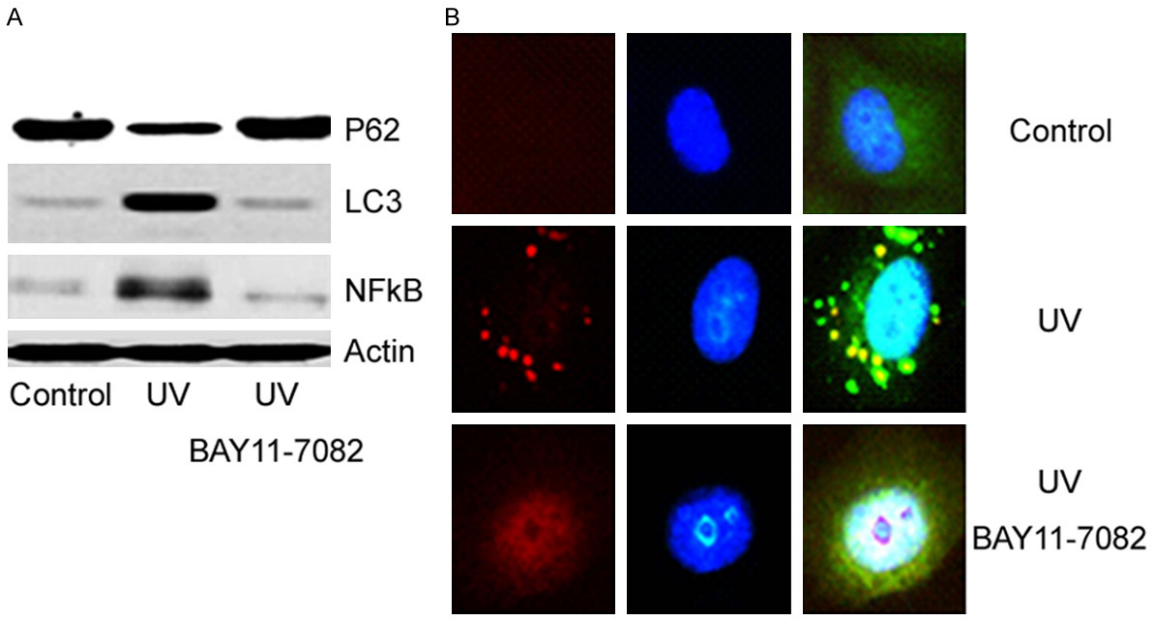

Figure 3. NF-κB signal pathway inhibitor BAY11-7082 suppressed UV-induced autophagy of RB cell line HXO-RB44. A. Western blot for cell autophagy; B. Confocal microscopy for cell autophagy, blue for P62 staining of nucleus, green for LC3 staining representing cell autophagy.

us one major challenge in medical science. Precise treatment is the ultimate solution for RB [6, 7], although the choice of targets is the major challenge. Therefore, it is urgent to discover more effective molecular targets for RB. More importantly, the treatment strategy for RB targeting NF-κB signal pathway is still lack [8, 9].

How does UV regulates RB cell growth and autophagy is still unclear [24]. NF-κB signal pathway can inhibit RB growth, but is correlated with tumor metastasis [25]. These indicated the possible involvement of NF-κB signal pathway in RB pathogenesis and progression [26].

NF-κB signal pathway is widely involved in cell autophagy. In current study, whether NF-κB signal pathway is under UV regulation for further mediation of autophagy of HXO-RB44 cells is still unclear [27, 28]. Results of this study showed that UV potentiated NF-κB signal pathway activity. After pre-treatment using NF-κB signal pathway activator, HXO-RB44 cells showed elevated autophagy level, whilst NF-κB signal pathway inhibitor suppressed UV-induced autophagy of HXO-RB44 cells. These results were consistent with previous study [10], proving that NF-κB signal pathway facilitated autophagy occurrence.

In this study, the role of NF-κB signal pathway in UV-induced autophagy or RB cell line HXO-RB44 can be demonstrated from three perspectives. Firstly, data showed significantly enhanced NF-κB signal pathway protein level in HXO-RB44 cells after UV irradiation. Secondly, pre-treatment using NF-κB signal pathway activator enhanced autophagy of HXO-RB44 cells after UV irradiation. Thirdly, when using NF-κB signal pathway inhibitor for pre-treatment, UV-induced autophagy of HXO-RB44 cells was suppressed. These results indicated the effect of NF-κB signal pathway protein on UV-induced autophagy of RB cell line HXO-RB44. Targeting NF-κB signal pathway protein thus might be a new strategy for molecular targeting treatment against RB [26]. Currently, NF-κB signal pathway also inhibits cancer cell autophagy in other tumors [28]. These results indicated that UV could induce RB cell line HXO-RB44 cell autophagy via enhancing NF-κB signal pathway.

Certain weakness existed in this study. Firstly, we lacked clinical tumor tissues and adjacent tissues of RB, making it impossible to investigate the direct relationship between NF-κB signal pathway activity and RB. Secondly, we did not include RB tumor tissues after treatment, thus cannot predict the correlation between NF-κB signal pathway activity and RB progno-

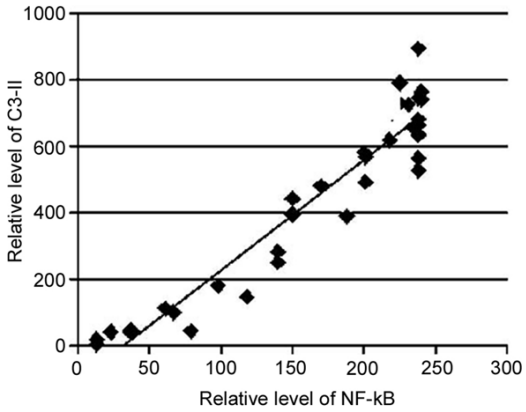

Figure 4. Correlation analysis between NF-κB signal pathway activity and autophagy level.

sis. Thirdly, no animal model of RB was used for UV irradiation, which may help to study the treatment efficiency of UV irradiation-induced RB in vivo.

Conclusion

UV can induce RB cell autophagy, which is possibly via NF-κB signal pathway, indicating that modulating NF-κB signal pathway activity might be beneficial for RB treatment through regulating autophagy level of RB.

Disclosure of conflict of interest

None.

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