Original Article
Experimental study of the RIP3 expression and cell death resistance in acute lymphoblastic leukemia Jurkat cells under TNF-α administration

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Abstract: The aim of this study was to investigate the expression pattern of receptor interacting protein kinase 3 (RIP3) in acute lymphoblastic leukemia (ALL) Jurkat cells under tumor necrosis factor alpha (TNF-α) administration, and to explore the underlying mechanism of Jurkat cells maintaining survival. After TNF-α administration (5, 10, 20, 40, 80 and 160 ng/ml), The cells viability, the cell death rate, the mRNA and protein level of RIP3 was analyzed. What we have found was that the mRNA and protein expression levels of RIP3 were upregulated under the stimulation of TNF-α, while the viability of Jurkat cells was inhibited and the cell death rate was increased. However, Jurkat cells still exhibited a high survival rate (65.23%) when TNF-α reached an extremely high concentration (40 ng/ml). Further study showed that at a TNF-α concentration approaching IC₅₀, the expression of RIP3 in Jurkat cells was upregulated significantly. Besides, the activity of caspase 8, the upstream initiator of the apoptosis cascade reaction, was increased as well, which resulted in increased cell death. On the contrary, inhibiting caspase caused a decrease in cell death. The RIP3 expression level was positively correlated to the degree of increase in caspase 8 activity and cell apoptosis rates. This indicated that RIP3 might activate the TNF-α-induced apoptosis of Jurkat cells via caspase 8. Moreover, under TNF-α treatment, NF-kB and autophagy activity were improved significantly. Inhibiting NF-kB and autophagy activity would promote cell death to a certain degree, indicating that NF-kB and autophagy might be an important pathway for the resistance of Jurkat cells against the TNF-α-induced apoptosis. It was through this pathway that the Jurkat cells avoided death and continued to survive. This hypothesis and the detailed mechanisms remain to be further investigated.

Keywords: TNF-α, RIP3, acute lymphoblastic leukemia, Jurkat cells, programmed cell death

Introduction
Acute lymphoblastic leukemia (ALL) is characterized by malignant proliferation of immature lymphocytes and immune disorders. In recent years, the incidence of ALL has increased because of the more serious levels of environmental pollution, which poses a serious threat to public health. Currently, chemotherapy remains to be the most important method for the treatment of ALL. With the development and application of new anti-leukemic drugs, the long-term disease-free survival of ALL patients has greatly improved. However, a poor response to chemotherapy has still been reported in about 30% of ALL patients. Therefore, drug resistance is a major cause of chemotherapy failure in ALL patients. Also, the complicated and diverse mechanisms of leukemic drug resistance have not been fully elucidated. But the recent studies have reported that the inhibition of apoptosis is one of the main mechanisms underlying multi-drug resistance in tumor chemotherapy.

It has recently been reported that necroptosis is a Caspase-independent type of PCD, which differs from traditional apoptosis and necrosis [1]. Necroptosis and apoptosis are similar in terms of their energy requirements and synthesis of new proteins.

Necroptosis is an active cellular process with self-regulation and occurs via transduction pathways that are similar to apoptosis; but both processes could be initiated by a combina-
tion of related death receptors and their ligands. After activation of the death receptor by its associated ligand, caspase-dependent apoptosis usually occurs. If the caspase activity is completely blocked, then the mode of cell death is altered from apoptosis to necroptosis [2]. Tumor cell apoptosis can be inhibited by Caspase inactivation, which does not affect necroptosis. Therefore, necroptosis provides a novel chemotherapeutic approach to tumors, which are resistance to anti-tumor drugs due to inhibition of apoptosis [3]. A large number of studies have found that both apoptosis and necroptosis are regulated by intracellular signaling molecules, and that RIP3 is a key molecule in the necroptosis signaling pathway and plays a central role in the switching from apoptosis to necroptosis [4, 5].

In recent years, numerous studies have focused on necroptosis of tumor cells, which may resolve the problem of drug resistance caused by the inhibition of apoptosis. Many agents have been shown to induce necroptosis in tumor cells successfully, which are unable to undergo apoptosis [6-8]. Recent studies have identified the reduced expression of RIP3 in acute myeloid leukemia (AML) cells and chronic lymphoblastic leukemia (CLL) cells, which inhibit both apoptosis and necroptosis [9, 10]. However, the expression pattern of RIP3 in ALL cells has not been documented. In addition, the association of RIP3 expression and PCD in ALL cells is not clear. In the present study, we investigated the expression pattern of RIP3 in ALL Jurkat cells, and to explore the underlying mechanism for Jurkat cells maintaining survival, which may provide a novel approach for the treatment of drug-resistant of ALL.

Materials and methods

Reagents

Rabbit anti-RIP3, Rabbit anti-LC3-II and P62 antibodies were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-P65 and anti-P-P65, GAPDH, RPMI-1640 medium containing 10% fetal bovine serum were purchased from Boster (Wuhan, China). A qPCR kit was purchased from Kapa Biosystems (Wilmington, MA, USA). The CCK8 kit was purchased from Dojindo Laboratories (Kumamoto, Japan). Alexa Fluor 488 Annexin V and a PI kit were purchased from KeyGEN BioTECH (JiangXu, China). TNF-α was purchased from Sigma (St. Louis, MO, USA). Jurkat cells were purchased from the cell bank of the Chinese Academy of Sciences. BAY 11-7082 (BAY), zVAD and 3-Methyladenine (3-MA) were all purchased from Selleck Chemicals (Houston, TX, USA).

Cell culture

Jurkat cells were maintained in the RPMI-1640 medium which containing 10% fetal bovine serum in an incubator with 5% CO₂ at 37°C. And the Cells had been grown to logarithmic growth phase for the analyses.

Cell viability assay

During the logarithmic growth phase, the Jurkat cells were taken and its concentration was adjusted to 2×10⁵/ml. Also, seven groups of Jurkat cells treated with different concentrations of TNF-α (0, 5, 10, 20, 40, 80 and 160 ng/ml) were established, and each group was assessed in triplicate. Jurkat cells cultured in RPMI-1640 medium without TNF-α (0 ng/ml) served as the control. After TNF-α treatment for 24 h, the proliferation of Jurkat cells was evaluated with the CCK8 kit according to the manufacturer’s instructions. The optical density (OD) of Jurkat cells at 450 nm was determined using a microplate reader (BIO-RAD, America) to analyze the rate of cell growth inhibition and the half maximal inhibitory concentration (IC₅₀). The Jurkat cells were then treated with the IC₅₀ of TNF-α, and the OD at 450 nm of the Jurkat cells after 0, 6, 12, 18 and 24 h of TNF-α treatment was determined to generate the cell growth curve.

Detection of programmed cell death

In the early stage of apoptosis, phosphatidyl serine (PS) shifts from the inner side to the outside of cell membrane. Annexin V is a Ca²⁺-dependent phospholipid-binding protein that specifically binds with PS high affinity. Using fluorescein-labeled Annexin V as a probe, flow cytometry can distinguish cells at early stage of apoptosis from dead cells (include middle/late stage of apoptosis cells and necrosis cells) when combined with PI staining [11]. In the present study, Jurkat cells were harvested after 24 hours of TNF-α treatment, 100 μl of which (1×10⁶/ml) were rinsed with PBS. After centrifugation, the cells were suspended
Expression of RIP3 in Jurkat cells

Staining buffer containing 5 μl of FITC-Annexin V and 1 μl of PI was used to evaluate cell death rate by flow cytometry after incubation for 15 min.

Evaluation of mRNA level of RIP3 in Jurkat cells

Total RNA was isolated from Jurkat cells using TRIzol RNA isolation kit. The mRNA level of RIP3 was evaluated using the forward primer 5'-CCAAA-TCCAGTACAGGGCG-3' and the reverse primer 5'-TCTTTAGGGCCTTCTTGCGA-3'. The internal control, β-actin, was amplified and analyzed using the forward primer 5'-AGCCTCGCCTTTGCCG-3' and the reverse primer 5'-CTCGGCCCCATAGGAAT-3'. The primers were designed with the ABI Primer Express 2.0 software and synthesized at Shanghai Sangon Biotech Company. The qRT-PCR was conducted using a FastStart Universal SYBRGreen PCR Master kit, and the samples were assessed in triplicate. The change in expression of RIP3 was evaluated using the 2^(-ΔΔCT) method.

Evaluation of protein level of RIP3 in Jurkat cells

Jurkat cells were rinsed three times with PBS, and total protein was isolated using protein isolation reagents. The protein concentration was determined using the Bradford colorimetric assay. After boiling in a water bath for three minutes, 50 μg of total protein per sample was separated by electrophoresis in a 10% SDS-PAGE. The proteins were then transferred to a PVDF membrane and blocked using 5% nonfat dry milk at room temperature for one hour. The proteins in the PVDF membrane were incubated with primary antibody at 4°C overnight. After rinsing three times with Tris-Buffered saline and Tween 20 (TBST), the PVDF membrane was incubated with the secondary antibody at room temperature for one hour. Finally, the PVDF membrane was rinsed three times with TBST and then examined using ECL chemiluminescence. The expression level of RIP3 protein was analyzed on an ImageQuant LAS 4000 mini imaging machine.

Investigate the influence of autophagy and NF-κB on the TNF-α induced PCD in Jurkat cells

Jurkat cells were divided into five groups. That is, TNF-α group, BAY group (TNF-α+10 μM BAY), 3-MA group (TNF-α+10 mM 3-MA), zVAD group (TNF-α+20 μM zVAD), the TNF-α concentration of these four groups was 20 ng/ml. Jurkat cells cultured in RPMI-1640 medium without TNF-α served as the control. Each group was assessed in triplicate. 2 h after treatment, cells were harvested, then the cell death rate was evaluated by flow cytometry, and Caspase 8 activity was determined by spectrophotometry. The protein level of RIP1, RIP3, P62, LC3-2, P65 and P-P65 were measured by Western blot. Based on the previous experiment.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). The t-test was used to evaluate the difference between groups. A P value less than 0.05 was considered statistically significant.

Results

TNF-α inhibited the viability of Jurkat cells

The results of the CCK8 assay showed that the viability of Jurkat cells was inhibited by TNF-α in a concentration-dependent manner. The maximal inhibitory effect was observed
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when the TNF-α concentration was increased to 40 ng/ml (Figure 1A). The calculated IC$_{50}$ was 14.96 ng/ml. Within 24 h, we observed an increasing inhibitory effect of the IC$_{50}$ of TNF-α on Jurkat cells with longer incubation times (Figure 1B). This finding indicated that, within a certain concentration range, TNF-α can inhibit Jurkat cells' viability to some extent.

**TNF-α increased cell death of Jurkat cells**

The flow cytometry results showed that TNF-α significantly increased apoptosis and necrosis of Jurkat cells compared with the control in a concentration-dependent manner. The rate of early apoptotic cells reached 18.04%, and the highest rate (9.91%) of late apoptotic/necrotic cells was obtained when the concentration of TNF-α was increased to 40 ng/ml (Figure 2A and 2B); however, 65.23% of the Jurkat cells still survived at this concentration of TNF-α (40 ng/ml) (Figure 2C). This finding indicated that in Jurkat cells, there must be some kinds of mechanisms to resist TNF-α induced cell death and maintain cell survival.

**TNF-α increased the expression of RIP3 in Jurkat cells**

Based on the qPCR results, TNF-α increased the expression of RIP3 in Jurkat cells. The highest level of RIP3 mRNA was detected in Jurkat

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*Figure 2. TNF-α induced apoptosis and programmed necrosis of Jurkat cells. A. Gated regions based on flow cytometry. B. Comparison of the rates of apoptosis and programmed necrosis. C. Comparison of the rates of surviving cells. *: $P < 0.05$, compared with the control group.*
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Figure 3. TNF-α increased the expression of RIP3 in Jurkat cells. A. TNF-α increased the mRNA level of RIP3 in Jurkat cells. B, C. TNF-α increased the protein level of RIP3 in Jurkat cells. *: P < 0.05, compared with the control group.

Influence of autophagy and NF-κB on the TNF-α induced PCD in Jurkat cells

As shown in Figures 4 and 5, under the treatment with 20 ng/ml TNF-α, the expressions of RIP1 and RIP3 were upregulated, especially the expression of RIP3. Moreover, the activity of caspase 8, the upstream initiator of the apoptosis cascade reaction, was positively correlated to the degree of increase in caspase 8 activity and cell death rates. Thus, RIP3 might be involved in the apoptosis of Jurkat cells through caspase 8. Our results also indicated that the LC3-2 level increased considerably under the treatment with TNF-α. There were no significant differences in P65 level across the groups. However, the expression of phosphorylated P65 (P-P65) was upregulated significantly, indicating an increase in NF-κB and autophagy activity. The use of autophagy and NF-κB inhibitor could promote cell death to a certain extent. In particular, inhibiting autophagy activity was more effective in promoting cell death rates. This indicated that autophagy may play a more critical role in maintaining survive in Jurkat cells.

Discussion

Apoptosis and necroptosis are two different types of cell death that are mediated by death receptors. Apoptosis relies on Caspase, and the activation of Caspase induces a protease cascade that culminates in the apoptosis of cells. Necroptosis is a Caspase-independent type of cell death. Both apoptosis and necroptosis are triggered by the binding of cell death ligands such as TNF-α and FasL to cell death receptors. In addition, apoptosis and necroptosis occur via similar signaling pathways, which involve RIP1 and RIP3. Interestingly, RIP3 plays an essential role in the transition and balance between apoptosis and programmed necrosis [11, 12].

Inhibition of apoptosis is one of the major mechanisms underlying the progress of tumor cells. Therefore, the induction of tumor cell apoptosis has extensive applications in chemotherapy for cancers. However, tumor cells tend to be resistant to apoptosis induced by anti-cancer drugs, significantly reducing the therapeutic effects of anti-cancer drugs [13-15]. As a Caspase-independent type of cell death, necroptosis may provide a new therapeutic strategy.
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Figure 4. Effects of inhibiting NF-κB, autophagy and Caspase activity on programmed cell death of Jurkat cells. BAY, inhibitor of NF-κB (10 µM); 3-MA, inhibitor of autophagy (10 m mol/l); zVAD, inhibitor of Caspase (20 µM). A. Gated regions based on flow cytometry. B. Comparison of the rates of apoptosis and programmed necrosis. C. Comparison of the rates of dead cells. *: $P < 0.05$, compared with the TNF-α treatment group.

death, necroptosis has the potential to be an effective strategy for the treatment of cancers that are resistant to apoptosis-inducing drugs. However, recent studies have demonstrated
that many tumor cells, including AML and CLL cells, are resistant to both apoptosis and necroptosis for the reduced expression of RIP3 [9, 10], suggest that reduced expression of RIP3 is associated with drug resistance of AML and CLL cells.

ALL, which accounts for 80% of acute leukemia in children, is the most common pediatric tumor. To date, the role and molecular mechanisms of RIP3 in ALL drug resistance have not been reported. In the present study, we examined the RIP3 expression pattern in Jurkat cells, and to explore the underlying mechanism for Jurkat cells maintaining survival, the aim of our study is to provide a novel approach for the treatment of drug-resistant of ALL.
Our study found that the expression of RIP3 was not down-regulated in Jurkat cells, on the contrary, the mRNA and protein expression levels of RIP3 in Jurkat cells increased significantly under the stimulation of TNF-α (5-160 ng/ml). This result indicated that, the RIP3 expression pattern in Jurkat cells is unlike that in AML and CLL cells [9, 10], there are no defect in the RIP3 expression of Jurkat cells. Therefore, the programmed cell death-promoting function of RIP3 in Jurkat cells may remained unaffected. The results of our study also showed that, under TNF-α stimulation, the viability of Jurkat cells was inhibited, and Jurkat cell death rate was increased, however, 65.23% of the Jurkat cells survived when the concentration of TNF-α was increased to 40 ng/mL, indicating that there might be other mechanisms leading to blockage of TNF-α induced cell death.

Both RIP1 and RIP3 are members of the RIP kinase family, which exhibit a strong pro-apoptotic effect [16, 17]. Our study indicated that the expressions of both RIP1 and RIP3 were upregulated at a TNF-α concentration closer to IC50. The upregulation was higher with RIP3. Compared with RIP1, RIP3 might have played a greater role in the TNF-α-induced apoptosis. Studies have demonstrated that caspase-2, -3, -7 and -10 are members of the RIP3-induced apoptotic pathway and that caspase-8 is the upstream initiator of the caspase cascade reaction [18, 19]. Our study demonstrated that as the expression of RIP3 was upregulated, the caspase 8 activity was increased as well, which resulted in an increased cell death rate. On the contrary, inhibiting caspase activity caused a decrease in the cell death rate, which proved that the caspase signaling pathway was involved in cell apoptosis. It was also observed that the RIP3 expression level was positively correlated to the degree of increase in caspase 8 activity and the cell apoptosis rate. This means RIP3 might activate the apoptosis of the Jurkat cells through caspase 8. However, more studies are needed to confirm this finding and to elucidate the working mechanism.

Ubiquitin-binding protein P62 is widely expressed in many cells and tissues and its expression is reversely correlated to autophagy activity. P62 is an indirect indicator of autophagy activity [23-25]. We found a significant upregulation of LC3-2 but a downregulation of P62 under TNF-α treatment. The expression of the later was negatively correlated to LC3-2 expression, indicating an increase in autophagy activity. P65 is an important member of the NF-κB family and a core transcriptional factor of this signal transduction pathway. NF-κB activation is the process where P-P65 is transported into the nuclei from the cytoplasm. Hence the changes in P-P65 expression relative to P65 can reflect the activity of NF-κB [26-28]. Our results indicated little changes in the P65 expression under TNF-α treatment, while the P-P65 level increased significantly, which was suggestive of an increased activity of NF-κB. Autophagy and NF-κB inhibitor could promote cell death to a certain degree, and inhibiting autophagy activity considerably increased the cell death rate, indicated that autophagy may play a more critical role in maintaining survive in Jurkat cells.

Taken together, we observed no defect in RIP3 expression in the Jurkat cells. In contrast, TNF-α treatment induced a considerable upregulation of RIP3 mRNA and protein, and RIP3 might be involved in the TNF-α-induced cell apoptosis. Our study also indicated that NF-κB and autophagy might be an important pathway by which Jurkat cells resisted the TNF-α-induced death. This hypothesis and the detailed mechanisms remain to be further investigated.

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Disclosure of conflict of interest

None.

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