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The autophagy molecule Beclin 1 maintains persistent activity of NF-κB and Stat3 in HTLV-1-transformed T lymphocytes

Li Chen [a, b, 1], Dan Liu [b, 1], Yang Zhang [b], Huan Zhang [b], Hua Cheng [b, c, d, e, *]

[a] Pharmacy College, Fujian University of Traditional Chinese Medicine, Fuzhou, China
[b] Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[c] Marlene and Stewart Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[d] Department of Medicine, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[e] Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201, USA

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ABSTRACT

The retroviral oncoprotein Tax from human T cell leukemia virus type 1 (HTLV-1) induces persistent activation of IκB kinase (IKK)/NF-κB signaling, an essential step for initiating HTLV-1 oncogenesis. The regulation of the IKK/NF-κB signaling in HTLV-1-transformed T cells remains incompletely understood. In the present study, we showed that the autophagy molecule Beclin1 not only executed a cytoprotective function through induction of autophagy but also played a pivotal role in maintaining Tax-induced activation of two key survival factors, NF-κB and Stat3. Silencing Beclin1 in HTLV-1-transformed T cells resulted in diminished activities of NF-κB and Stat3 as well as impaired growth. In Beclin1-depleted cells, Tax failed to activate NF-κB and Stat3 at its full capacity. In addition, we showed that Beclin1 interacted with the catalytic subunits of IKK. Further, we observed that selective inhibition of IKK repressed the activities of both NF-κB and Stat3 in the context of HTLV-1-transformation of T cells. Our data, therefore, unveiled a key role of Beclin1 in maintaining persistent activities of both NF-κB and Stat3 in the pathogenesis of HTLV-1-mediated oncogenesis.

1. Introduction

Human T cell leukemia virus type 1 (HTLV-1) is the only human retrovirus that is etiologically linked to adult T cell leukemia and lymphoma (ATL) [1]. The viral genome of HTLV-1 encodes a transforming protein, named Tax, which plays a central role in transforming CD4+ T lymphocytes [2]. Expression of Tax is crucial not only for transactivating viral gene transcription but also for promoting survival and proliferation of virally infected human T lymphocytes [3,4]. Through dysregulation of cellular oncogenic signaling pathways, Tax promotes cell cycle progression, leading to aberrant proliferation of HTLV-1-infected T cells [4]. Notably, Tax stimulates IκB kinase complex (IKK), resulting in persistent activation of NF-κB [5]. Blockade of IKK/NF-κB signaling causes apoptosis of HTLV-1-transformed T cells, supporting the notion that the constitutive activation of NF-κB is a prerequisite for HTLV-1 transformation of T cells [6].

We have shown that Tax deregulates autophagy through activation of IKK, and this process is crucial for maintenance of HTLV-1-mediated T cell malignancy [16]. Tax directly targets the Beclin1-containing autophagy molecular complex to increase autophagic flux, while silencing Beclin1 affects survival and proliferation of HTLV-1-transformed T cells [7]. Autophagy is initiated to degrade aggregated cellular proteins or aged organelles through the autophagosome-lysosome degradation pathway, providing valuable energy source for cells under nutrient deprivation or other stress conditions [8]. Autophagy also plays key roles in anti-inflammation, viral infection, neurodegenerative disorder, autoimmune disease and tumor suppression by maintaining chromosomal integrity [8]. In certain contexts and stages of cancer, autophagy can function to promote tumor progression and metastasis [9].

Beclin1 is one of the key components of the Beclin1-PI3 kinase class III protein complex that mediates vesicular nucleation during formation of autophagosome [8]. In addition to the interaction of Tax with Beclin1, other viral proteins target at Beclin1 by either

* Corresponding author. Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD 21201, USA.
E-mail address: hcheng@ihv.umaryland.edu (H. Cheng).
[1] These authors contribute equally.

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stimulating or inhibiting its activity [7,10]. Beclin1 is transcriptionally upregulated by NF-κB and is required for tumor necrosis factor alpha (TNFα)-mediated activation of NF-κB [11,12]. Given the observation that a persistent activation of NF-κB is essential for HTLV-1 oncogenesis, we reason that Beclin1 may play a role in NF-κB signaling in HTLV-1-transformed T cells. In the present study, we have shown that Beclin1 is crucial for maintaining constitutive activation of NF-κB and Stat3 induced by Tax.

2. Materials and methods

2.1. Cell lines, antibodies and chemicals

MT-2 and Jurkat cell line were obtained from AIDS research and reference reagent program (NIAID, National Institutes of Health). SLB-1 and MT-1 cell lines were described previously [7]. These cells were cultured in RPMI1640 medium supplemented with 10% FBS. HEK293 cells were cultured in DMEM medium containing 10% FBS.

Antibodies for IKKα, IKKβ, IKKγ, Beclin1, SQSTM1/p62, HA and GST were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for LC3, Atg5, PI3KC3, and cleaved caspase 3, 7, 9 and PARP were from Cell Signaling (Danvers, MA, USA). Anti-β-actin and anti-Flag were from Sigma (St Louis, MO, USA). Monoclonal anti-Tax antibody was obtained from AIDS Reagent Program. DMSO, U0126, LY294002 and BAY11-7805 were purchased from Sigma (St Louis, MO, USA). APC-Annexin V and 7-AAD staining solution were purchased from BD Biosciences (San Jose, CA, USA).

2.2. Plasmids, immunoblot, glutathione S-transferase (GST) pulldown assay

The expression plasmids for GST-tagged IKKα, IKKβ, IKKγ, Beclin1 and HA-tagged Tax, M22 and M47 as well as Flag-tagged Beclin1 and PI3KC3 were described previously [7]. The mutants of Beclin1 were generated using a PCR-based mutagenesis method. Lentivirus vectors for IKKα- and IKKβ-specific shRNAs were described previously [7], and the lentivirus vectors for IKKγ- and Beclin1-specific shRNAs were purchased from Open Biosystems (Pittsburgh, PA, USA). Lentivirus vector for PI3KC3-specific shRNA was purchased from Thermo Scientific (Grand Island, NY, USA). Western Blot (WB), co-immunoprecipitation and GST pulldown assays were performed as described previously [7].

2.3. Electrophoretic gel mobility shift assay (EMSA)

Nuclear extracts were prepared from various cell lines using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, IL, USA). The oligonucleotides were 5'-end labeled with biotin (Integrated DNA Technologies, Coralville, IA, USA) and annealed to their complementary strands. The binding activities were examined by EMSA using Light Shift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA). The sequences of the probes for NF-κB, Stat3, AP-1 and OCT1 were previously reported [7].

2.4. Cell viability assay by trypan blue exclusion and FACS

Cells were transduced with recombinant lentiviruses that were produced and concentrated as previously described [41]. Three days post-transduction, cells were collected and re-suspended in fresh complete medium. An aliquot of cells was added to an equal volume of trypan blue (0.4%). The viable cells were measured by trypan blue exclusion assay. Each experiment was conducted in triplicate and the results were indicated as the mean ± SD.

The induction of apoptosis was determined using an APC-Annexin V/7-AAD double staining. NS (non-specific)-, Beclin1-depleted MT-2 and SLB-1 cells were harvested and washed twice with PBS buffer 6 days post-transduction. The cells were resuspended with 100 μl binding buffer at 1×10^6 cells/ml, 5 μl of APC-Annexin V and 5 μl of 7-AAD were added and incubated for 15 min at room temperature at dark. The results were analyzed by flow cytometry.

3. Results

3.1. Silencing Beclin1 leads to growth arrest and apoptosis of HTLV-1-transformed T cells

To determine the role of the autophagy molecules in promoting aberrant proliferation of HTLV-1-transformed T cells, we applied a lentiviral delivery of specific shRNA method to silence selected autophagic molecules including Beclin1, PI3KC3 and Atg5. We employed four model T cell lines including MT-2 and SLB-1 cell lines (HTLV-1-transformed human CD4+ T cells that express Tax), MT-1 cell line (adult T cell leukemia cells with lack of Tax expression) and Jurkat T cell line (non-HTLV-1-infected, CD4+ lymphoblastic leukemia cells). We found that knockdown of either Beclin1 or Atg5 in SLB-1 cells resulted in impaired survival and proliferation, and the growth of the Atg5-depleted cells were affected more severely (Fig. 1A and B). Similarly, silencing the key autophagy molecule Beclin1, Atg5 or PI3KC3 in MT-2 cells led to growth arrest (Fig. 1C). In contrast, depletion of either Beclin1 or Atg5 had no apparent growth impairment in MT-1 and Jurkat T cells (Fig. 1D and E). We found that silencing Beclin1 resulted in an increased annexin V staining in MT-2 and SLB-1 cells (Fig. 1F). Further, depletion of Beclin1 led to caspase-dependent apoptotic cell death as evidenced by increased production of the cleaved forms of caspase-3, -7, -9 and PARP (Fig. 1G). These findings support a key role of Beclin1 in promoting survival of HTLV-1- transformed T cells.

3.2. Beclin1 is required for tax activation of NF-κB and Stat3

Maintenance of an optimal NF-κB activity is essential for survival and proliferation of HTLV-1-transformed T cells, particularly at early stage of oncogenesis. To elucidate a potential role of the autophagy molecules in regulating NF-κB activity, we silenced Beclin1, Atg5 or PI3KC3 with lentiviral transduction of the specific shRNAs in HTLV-1-transformed T cells. We found that depletion of Beclin1, Atg5 or PI3KC3 did not affect the protein levels of the IKK complex containing three subunits, IKKα, IKKβ and IKKγ as well as Tax (Fig. 2A). Next, we examined the expression of the autophagy marker proteins LC3 and p62/sequestosome1. LC3-II, the lipidated form of LC3-I, is generated during autophagy process, exhibiting a slightly faster mobility than LC3-I on SDS-PAGE. p62 is a master regulator of autophagy, which functions to conjugate ubiquitinated cellular proteins for targeting them into the autophagosome-lysosome degradation pathway. We observed that the LC3-II protein was accumulated in Beclin1-or PI3KC3-depleted T cells whereas it was reduced in Atg5-depleted cells (Fig. 2A and B). This result was consistent with the role of Atg5 at the early stage of vesicular nucleation during autophagy in which LC3-I was processed to generate the lipidated LC3-II. Silencing Beclin1 or PI3KC3 blocked fusion of autophagosome with lysosome, thereby preventing degradation of LC3-II. In contrast, p62 was accumulated in the autophagy molecule-depleted MT-2 and SLB-1 cells due to the blockade of autophagic flux (Fig. 2A and B).

We determined if inhibition of autophagy affects the activity of NF-κB in HTLV-1- transformed T cells expressing Tax. We found that in Beclin1-, Atg5-or PI3KC3-depleted SLB-1 and MT-2 cells, the activity of both NF-κB and Stat3 was diminished (Fig. 2A and B). AP-1 was highly activated in HTLV-1-transformed T cells and its activity

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