Original Article

Evaluation of autophagy induction and inhibition in the Huh7.5 cell line through flow cytometry

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Abstract

Background: Autophagy is a physiologic process in which double membrane vesicles engulf damaged proteins and organelles for delivering them to lysosome in order to degrade and recycle them via lysosomal digestion. Beclin1 is one of the basic proteins involved in the initial step of auto phagosome formation. In the current study, the effects of exogenous Beclin1 and 3-methyladenine (3-MA) to induce and inhibit of autophagy were assessed in Huh7.5 cells as an in vitro models of hepatocellular carcinoma. **Materials and Methods:** The recombinant pcDNA-Beclin1was transfected into Huh7.5 cells. Also, the cytotoxicity of 3-methyladenine (3-MA) were determined in Huh7.5 cells and the cells were treated with 3-methyladenine (3-MA). The autophagy induction and inhibition was conducted via LC3 staining as a main autophagy marker using flowcytometry. **Results:** The result of this study suggest that the over expression ofexogenousBeclin1 in Huh7.5 cells elevated the autophagosome formation as shown by intracellular autophagosomal marker LC3-II staining for about 32.32 % and 3-MA decreased it up to2% compared with control cells in which the stained LC3-II was12.08.The IC50 of 3-methyladenine (3-MA) was 7.2 mM in Huh7.5 cells. **Conclusion:** Recombinant beclin1 may be used as a potential autophagy inducer agent and3-methyladenine (3-MA) inhibits autophagy formation in Huh7.5 cell. The staining autophagy formation marker LC3-II with specific antibody is a reliable method to measure autophagy activation via flow cytometry.
Keywords: Autophagy, Flow cytometry, LC3-II.

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Introduction

Autophagy is a preserved intracellular homeostatic process by which the cell cleans out different types of cytoplasmic debris(1). During the autophagy process, Beclin-1 and microtubule-associated protein 1 light chain3 (LC3) are connected with the initial steps of autophagosome formation and autophagosome maturation, respectively(2). To start autophagy, Beclin 1 is an initiator complex that acts as a platform for nucleation step of autophagy. One of the key biological markers frequently used to show autophagy maturation in mammalian systems is the microtubule associated protein 1A/1Blight chain 3 (LC3). During elongation of the phagophore, cytosolic LC3-1 is conjugated to phosphatidylethanolamine to form LC3-II. LC3-II is recruited and inserted into the autophagosomal membrane(3). Finally, the delivery of autophagosomes to lysosomes and degradation takes place(4).

Failure in autophagy causes the accumulation of intracellular macromolecules including unfolded and

damaged proteins, damaged organelles and chromatin instability DNA .The most important mark to show the role of autophagy in tumor suppression is a study carried out on the interaction between Bcl2 and Beclin 1 proteins. Deletion of Beclin1 has been mounted in prostate cancer, breast, ovarian both in humans and mice. Activation of PI3K-AKT-mTORpathwaythat inhibits autophagy is a dominant condition for cancer culminating in tumor cell survival and proliferation. In contrast, tumor-suppressor proteins such as RB, P53 and phosphates have a positive role on autophagy formation(5).

Hepatocellular carcinoma (HCC) is an aggressive form of cancer and is the fifth most common cause of cancer death worldwide(6).The interaction between apoptosis and autophagy is remained as debated issue particularly in HCC. Hence, molecular mechanisms contributed to chemo resistance of HCC may result in improved clinical results. Among the several parameters , therapy-induced autophagy indicate a novel approach of resistance to combat against cancer(7).

Throughout dysplastic stage in hepatocytes, basal autophagy able to suppress tumor suppressor via eliminating newly damaged mitochondria and gnomically unstable cell to maintain genomic stability. Autophagy inhibitors lead to tumor suppression in tumor-forming stage in the HCC rat model, but result in tumor-promotion in dysplastic phase. For this reason, the autophagy plays like two edge sward in the generation and progression of HCC based on the context of liver cells(8).

Interestingly, evidence has been mounting that autophagy itself may be another cell death mechanism, termed programmed cell type II or autophagic cell death (ACD), emphasizing a potential pro-death role for autophagy ACD is characterized by the accumulated cytoplasmic vacuolization, absence of chromatin condensation, LC3 lipidation and caspase-independent apoptosis(9).

Huh7.5 is derivative of Huh-7 cells (is a human hepatocyte-derived carcinoma cell line) that shows significantly superior permissiveness to replication of HCV. Huh7.5 cells are the RIG-I pathway deficient cells, making them less sensitive to intracellular dsRNA throughout virus replication and eliciting an antiviral response(10).The manipulation of autophagy in Huh7.5 can aid to clear the cross-talk between intracellular innate responses and viral immunopathogenesis. The aim of this study was to optimize the autophagy induction with recombinant Beclin and autophagy inhibition using 3-methyladenine (3-MA) by staining the intracellular autophagosomal marker LC3-II using flow cytometry(11) .

Methods

Plasmid construction. The recombinant plasmid pcDNA3.1 containing Beclin 1was gifted by Dr. Asghar Abdoli (Pasteur Institute of Iran).The vector was transferred into Escherichia coli(E. coli) strain DH5a and the plasmid was purified from an overnight culture of E. coli by using Miniprep Kit (Genetbio, Korea).(The Victor is 4,000 bp and the Beclin-1 gene is 1600 bp).The quality and concentration of purified plasmid DNA were controlled using agarose gel electrophoresis together with measuring optical density (OD) of DNA at wave lengths of 260 nm and 280 nm.

MTT assay and IC50 determination. Autophagy inhibitor, 3-methyladenine (3-MA) is a synthetic compounds and a cell permeable autophagic blocker through the inhibition of class III PI3K.To evaluate the toxicity of 3-methyladenine (3-MA) on cell viability, colorimetric MTT assay was carried out. Yellow3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), a water-soluble dye that is readily taken up by viable cells and reduced to purple formazan through mitochondrial succinate dehydrogenase. Briefly, to make stock MTT (10x), tetrazolium at a concentration of 5 mg/ml was dissolved in PBS (Phosphate Buffer Saline) and filtering via 0.45 µm filter. The medium of the treated cell with different 10 fold diluted 3-methyladenine (3-MA) was replaced with 100 µl of 1x MTT per well. After incubation at 37°C with 5% CO2 for 4 h, formazan crystals were dissolved in 100 µl dimethyl sulfoxide and shaked to release the color from the cells. Finally, Optical density was measured at 570 nm and 630nm using ELISA reader to determine live cells(12).

Cell culture and treatment. One day before transfection 2 × 105 Huh7.5 cells were seeded into in

six-well culture plates in DMEM supplemented with 10% fetal calf serum 100 IU/ml penicillin and 100 mg/ml streptomycin and incubated in the presence of 5 % CO2 incubator. After reaching 80% confluency, the growth medium was removed from cells and washed for two times with PBS buffer. In the next step, cells were transfected with 5µg of pcDNA-beclin 1 for expression of Beclin 1 or the empty vector as control using lipofectamine 2000(Sigma Company, Germany) based on manufacturer protocol. Also, to inhibit autophagy, Huh-7.5 cells were treated with different concentrations of 3-methyladenine (3-MA) ranging from 1 to 10 mM of 3-methyladenine (3-MA)(13).

Detection of autophagy induction by flow cytometry. Tracking the LC3-I to LC3-II conversion is indicative of autophagic activity which is recruited to autophagosomal membranes. LC3-II is one of the central proteins of the autophagosome membrane that is present in both the inner and outer site of the autophagosome membrane. In the current study, activation of autophagy was verified via autophagosomal marker LC3-II detection using specific antibodies and flow cytometry. Briefly, after fixation with 4% formaldehyde for 15 min and washing with PBS, the monolayer cells were permeabilized by adding 0.1% Triton X-100 in PBS for 15 minutes at 37° C. After removing Triton, the cells were covered by primary antibody diluted in PBS containing 1% BSA for 1h on ice. Finally, FITC-conjugated secondary antibody (Abcam, USA) was added and incubated at room temperature for 1h. The stained cells were studied by flow cytometry(Partec, Germany).Cells transfected with pcDNA empty vector were used as the negative control(14).

Statistical analysis. To provide data, the measures of central tendency were calculated by using mean for each well.

Results

Cell culture. Huh7.5 cells reached the desired confluency in terms of number and morphology after passages were shown in Figure 1.

MTT assay and IC50 determination. The percentage of the viability cells against different concentration of 3-methyladenine (3-MA) using

Figure 1. Inverted microscopic image of Huh7.5 cells after 48 hours cultivation in DMEM with 10% serum with a magnification of 40X

MTT assay was determined. Figure 2 shows the viability of Huh7.5 cells following exposure to different concentrations of 3-methyladenine (3-MA).The IC50 values of 3-methyladenine (3-MA) are obtained from the dose response curves (figures 3).The IC50 value of 3-methyladenine (3-MA) was 7.3 mM.

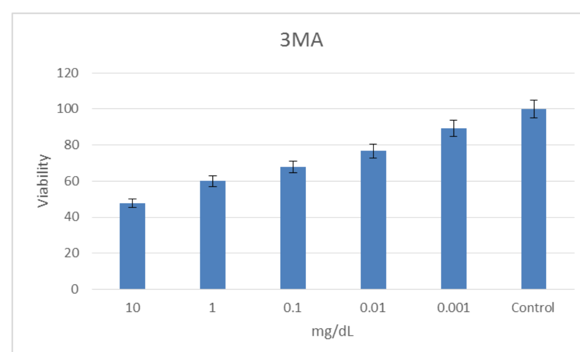

Figure 2. Cell Viability of Huh7.5 cells after 24 hours treatment with different concentrations of 3-methyladenine (3-MA).

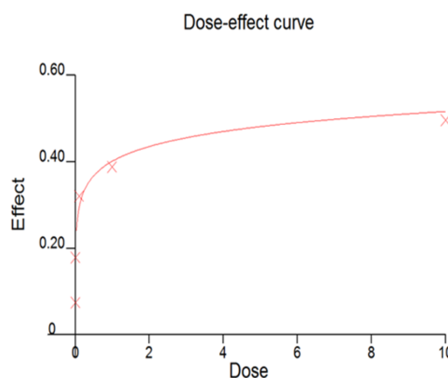

Figure 3. The dose–effect curve of 3-methyladenine (3-MA).

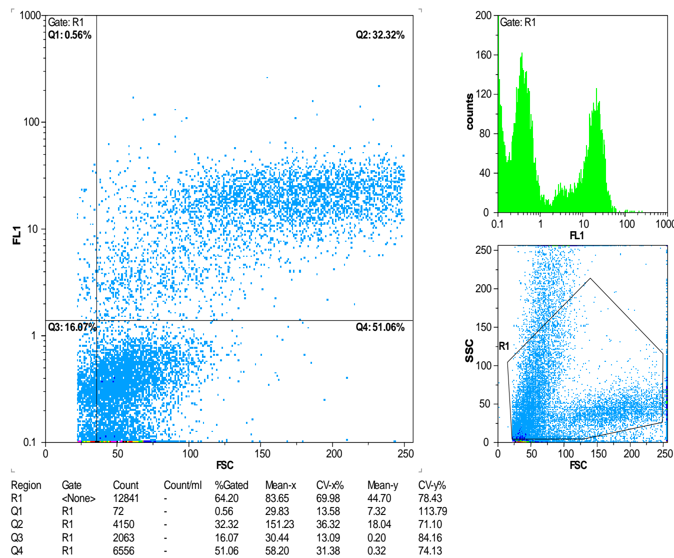

Figure 4. Autophagy induction by recombinant Beclin1.The staining intracellular marker of autophagy formation demonstrated that the 32.32 % of the cells were LCII positive when transfected with pCDNA-Beclin1 as autophagy inducer construct.

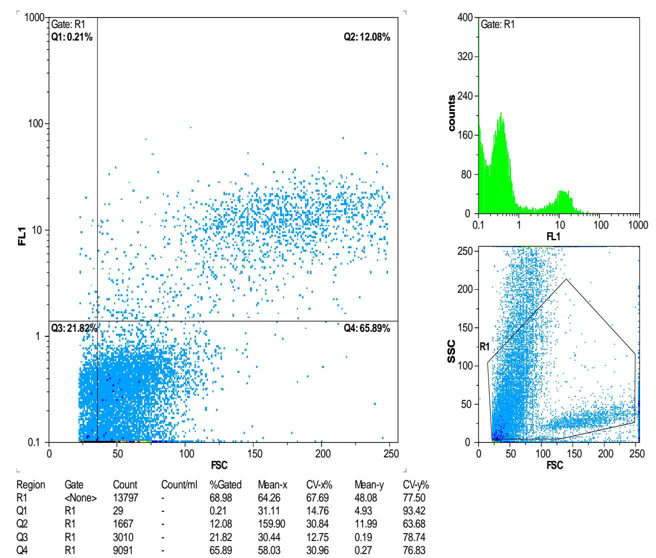

Figure 5. Autophagy evaluation after empty pCDNA3.1 plasmid transfection as a control. The Huh 7.5 cells were transfected with empty pCDNA3.1 stained with specific antibody to determine LC3II positive cells. Approximately, 12.08 % of cells were LC3II positive after staining.

Induction of autophagy in Huh7.5 by exogenous Beclin 1. LC3 is the one of main reliable marker of autophagosomes formation. We tested the ability of Beclin 1 to trigger autophagy in Huh7.5 cells using monoclonal antibody through staining LC3-II. As can be seen in Figure 4, 32.32 % of the cells were LC3-II positive in the experiment sample in comparison with the LC3-II expression level in control cell which was 12.08%, as shown in Figure 5.

Autophagy inhibition via 3MA. 3-Methyladenine (3-MA) is an autophagy inhibitor and selectively blocks PI3K.So we tested the capability of 3-Methyladenine (3-MA) in inhibition of autophagy in Huh7.5 cells. It reduced autophagy formation up to 2.2%in comparison with the LC3-II expression level in control cell which was 12.08%, as demonstrated in Figure 6.

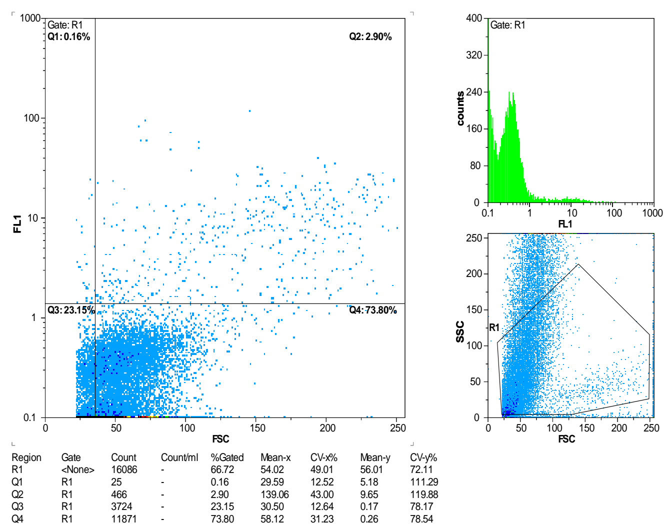

Figure 6. Autophagy inhibition using 3-methyladenine (3-MA).The class III PI3K blocker 3-methyladenine (3-MA) is used to inhibit autophagy activation and it reduced autophagy formation up to 2.80 %.

Discussion

In cancer cells, metabolic stress is because of inadequate nutrition or decreased storage oxygen and increased require for quick cell division, induced autophagy because of cell search for energy source and metabolism alternatives. Also, autophagy may be a response to acquired cancer cells in cancer treatments which leads to drug resistance and cancer cell survival. Suppression of autophagy via drugs including3-methyladenine (3-MA) make the cancer cells sensitive to a variety range of therapeutic models(15).

Parallel with this study, Kabeya Y and colleagues reported that conversion of LC3-I to LC3-II was repressed by 3-methyladenine (3-MA)(16). These results verified the autophagy inhibitory effects of 3-MA under normal conditions. In line with our studies, Tajo and et al. showed that Beclin 1 overexpression

induces autophagy in HeLa cells. They observed a number of LC3-positive cells by flow cytometry analysis in HeLa cells transfected with pcDNA-Beclin 1. The Beclin 1 overexpression effect on autophagy formation in transfected cells was more than that of the control group. In total, 51.7% of the cells were LC3-II positive in the experiment samples(17).

LC3 immunostained cells showed fewer numbers of punctuated stained cells on 3-methyladenine (3-MA) plus I2 treatment as compared to I2 treatment alone, suggesting disruption of autophagosome formation. In addition, inhibition of autophagy with PI3K (3-MA, LY294002) or H+/ATPase (bafilomycin) inhibitors enhance the cytotoxic response of I2. These data suggest that autophagy is acting as survival mechanism while extensive damage may be responsible for cytotoxic response in I2 treated cancer cells(18).

Differential effects of the autophagy inhibitors 3-methyladenine (3-MA) and chloroquine on the control of autophagy on HL60 cancer cells, was shown that treating with 3-methyladenine (3-MA),showed no significant change in the LC3-ll bands using Western blotting, while in the cells incubated with chloroquine, a significant increase in the expression of this protein was observed. Based on these observations, it is hypothesized that depending on the stage of the autophagy process that is targeted for the drug, the type of response to the drug in the amount of expression of various genes involved in autophagy can be different(19).

Demishtein A and et al., were reported when chloroquine or bafilomycin A1 are cultured separately with neuronal coronal cells lead to autophagy inhibition by measuring and observing the LC3-ll protein(20).3-methyl adenine(3-MA) can suppress proteolysis even in Atg5-deficient cells, suggesting that its effects on protein degradation extend beyond its role in autophagy inhibition(21).

In this study, after treating the Huh7.5 cells with 3-methyladenine (3-MA), the expression of LC3-ll was measured by flowcytometry and it decreased autophagy marker compared to the control cell. To induce autophagy, Beclin1 transfected Huh7.5 cells showed significant increases in expression of LC3-ll, compared to control cells.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Kudchodkar SB, Levine B. Viruses and autophagy. Reviews in Medical Virology. 2009;19(6):359-78.
2. Sir D, Tian Y, Chen W-l, Ann DK, Yen T-SB, Ou J-hJ. The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. Proceedings of the National Academy of Sciences. 2010;107(9):4383-8.
3. Tanida I, Ueno T, Kominami E. LC3 and Autophagy. Autophagosome and Phagosome. 2008:77-88.
4. Geng J, Klionsky DJ. The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. EMBO Reports. 2008;9(9):859-64.
5. Lorin S, Hamaï A, Mehrpour M, Codogno P, editors. Autophagy regulation and its role in cancer. Seminars in Cancer Biology; 2013: Elsevier.
6. Llovet JM, Bruix J. Molecular targeted therapies in hepatocellular carcinoma. Hepatology. 2008;48(4):1312-27.
7. Kang M, Jeong CW, Ku JH, Kwak C, Kim HH. Inhibition of autophagy potentiates atorvastatin-induced apoptotic cell death in human bladder cancer cells in vitro. International Journal of Molecular Sciences. 2014;15(5):8106-21.
8. Sun K, Guo X, Zhao Q. Paradoxical role of autophagy in the dysplastic and tumor-forming stages of hepatocarcinoma development in rats. Cell Death & Disease. 2013;4(2):e501.
9. Zhang M, Su L, Xiao Z, Liu X, Liu X. Methyl jasmonate induces apoptosis and pro-apoptotic autophagy via the ROS pathway in human non-small cell lung cancer. American Journal of Cancer Research. 2016;6(2):187.
10. Bartenschlager R, Pietschmann T. Efficient hepatitis C virus cell culture system: what a difference the host cell makes. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(28):9739-40.
11. Marino G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: the interplay of autophagy and apoptosis. Nature Reviews Molecular Cell Biology. 2014;15(2):81.
12. Van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Methods in Molecular Biology (Clifton, NJ). 2011;731:237-45.
13. Blight KJ, McKeating JA, Marcotrigiano J, Rice CM. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture.

Journal of Virology. 2003;77(5):3181-90.
14. Taji F, Kouchesfahani HM, Sheikholeslami F, Romani B, Baesi K, Vahabpour R, et al. Autophagy induction reduces telomerase activity in HeLa cells. Mechanisms of Ageing and Development. 2017;163:40-5.
15. Eng C, Abraham R. The autophagy conundrum in cancer: influence of tumorigenic metabolic reprogramming. Oncogene. 2011;30(47):4687-96.
16. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. The EMBO Journal. 2000;19(21):5720-8.
17. Feizi N, Mehrbod P, Romani B, Soleimanjahi H, Bamdad T, Feizi A, et al. Autophagy induction regulates influenza virus replication in a time-dependent manner. Journal of Medical Microbiology. 2017;66(4):536-41.
18. Singh P, Godbole M, Rao G, Annarao S, Mitra K, Roy R, et al. Inhibition of autophagy stimulate molecular iodine-induced apoptosis in hormone independent breast tumors. Biochemical and Biophysical Research Communications. 2011;415(1):181-6.
19. Palmeira-dos-Santos C, Pereira GJ, Barbosa CM, Jurkiewicz A, Smaili SS, Bincoletto C. Comparative study of autophagy inhibition by 3MA and CQ on Cytarabine-induced death of leukaemia cells. Journal of Cancer Research and Clinical Oncology. 2014;140(6):909-20.
20. Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. Cell. 2010;140(3):313-26.
21. Mizushima N, Yamamoto A, Hatano M, Kobayashi Y, Kabeya Y, Suzuki K, et al. Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. The Journal of Cell Biology. 2001;152(4):657-68.