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Autophagy induction regulates influenza virus replication in a time-dependent manner

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Abstract

Purpose. Autophagy plays a key role in host defence responses against microbial infections by promoting degradation of pathogens and participating in acquired immunity. The interaction between autophagy and viruses is complex, and this pathway is hijacked by several viruses. Influenza virus (IV) interferes with autophagy through its replication and increases the accumulation of autophagosomes by blocking lysosome fusion. Thus, autophagy could be an effective area for antiviral research.

Methodology. In this study, we evaluated the effect of autophagy on IV replication. Two cell lines were transfected with Beclin-1 expression plasmid before (prophylactic approach) and after (therapeutic approach) IV inoculation.

Results/Key findings. Beclin-1 overexpression in the cells infected by virus induced autophagy to 26 %. The $\log_{10}$ haemagglutinin titre and $TCID_{50}$ (tissue culture infective dose giving 50 % infection) of replicating virus were measured at 24 and 48 h post-infection. In the prophylactic approach, the virus titre was enhanced significantly at 24 h post-infection ($P \leq 0.01$), but it was not significantly different from the control at 48 h post-infection. In contrast, the therapeutic approach of autophagy induction inhibited the virus replication at 24 and 48 h post-infection. Additionally, we showed that inhibition of autophagy using 3-methyladenine reduced viral replication.

Conclusion. This study revealed that the virus (H1N1) titre was controlled in a time-dependent manner following autophagy induction in host cells. Manipulation of autophagy during the IV life cycle can be targeted both for antiviral aims and for increasing viral yield for virus production.

INTRODUCTION

Autophagy is a conserved intracellular homeostatic process by which the cell cleans out various cytoplasmic debris [1]. Autophagy is induced by three main pathways: chaperone-mediated autophagy, microautophagy and macroautophagy. Macroautophagy plays a key role in virus replication by wrapping virus with double membranes to force its removal by fusion with lysosomes and degradation by hydrolases and proteases [2, 3]. During the autophagy process, Beclin-1 and microtubule-associated protein 1 light chain 3 (LC3) are involved in the initial steps of autophagosome formation and autophagosome maturation, respectively. When a signal triggers autophagy through the Beclin-1 complex, cellular LC3 protein switches from its non-lipidated cytosolic LC3-I form to a phosphotidylamine-conjugated LC3-II form attached to the autophagosome membrane [4–6]. Several studies have shown autophagy induction by monitoring the transformation of LC3-I to LC3-II [4]. For antiviral functions, the viral components or virions are engulfed in autophagosomes for lysosomal degradation [5, 7–10]. However, some viruses hijack the components of the autophagic machinery in favour of their own replication or to egress from infected cells. For instance, herpes simplex virus 1, mouse herpesvirus 68 (MHV-68) and Kaposi's sarcoma-associated herpesvirus

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Abbreviations: 3-MA, 3-methyladenine; FBS, fetal bovine serum; HA, haemagglutinin; IAV, influenza A virus; IV, influenza virus; MDCK, Madin–Darby canine kidney; RT, room temperature; $TCID_{50}$, tissue culture infective dose giving 50 % infection; TPCK, tosyl phenylalanyl chloromethyl ketone.

inhibit autophagosome formation to escape autophagic degradation [6]. However, human immunodeficiency virus 1, poliovirus and mouse hepatitis virus block the degradation of autophagosomes and use them as a platform to assemble their RNA complexes to increase viral yield [2]. Thus, autophagy plays both antiviral and pro-viral roles in the course of virus infection and the pathogenesis of viruses.

It is essential that the interactions of influenza virus (IV) and its host are identified in order to carry out efficient countermeasures to inhibit viral infection. IV encounters autophagy during its life cycle and interacts with many cellular proteins in this process. Recently, it was shown that autophagy is induced by infection with influenza A virus (IAV) and serves as a crucial factor for viral proliferation, including assembly of the viral components throughout the life cycle of IAV [11]. In contrast, IAV hinders autophagosome formation through interaction with signalling pathways and autophagy-related genes. In addition, it was reported that IAV-induced apoptotic cell deaths were significantly enhanced in autophagy-deficient cells [12]. Interestingly, highly pathogenic strains of IAV, such as H5N1, induce autophagic cell death and interact with the autophagy machinery using mechanisms different from those utilized by weakly pathogenic strains [13, 14].

In this study, we analysed the effects on virus titre of targeting the macroautophagy pathway. Beclin-1, as a prominent macroautophagy initiator, was overexpressed to increase autophagosome formation. We transfected cells with Beclin-1-expressing plasmid before and after IAV inoculation. This allowed us to induce autophagy and to evaluate its effect on IAV titre in two cell lines: Madin–Darby canine kidney (MDCK) and MDCK-SIAT1. In addition, autophagy formation was blocked after virus inoculation using 3-methyladenine (3-MA) as an autophagy inhibitor.

METHODS

Reagents and chemicals

Lipofectamine 3000, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), antibiotics and trypsin were obtained from Invitrogen. The 3-MA was purchased from Sigma-Aldrich. LC3-II and goat anti-rabbit FITC-conjugated IgG antibodies were purchased from Abcam. The plasmid DNA extraction kit was from Yekta Tajhiz Azma. Gene synthesis was conducted by Eurofins. The MDCK cell line was obtained from the National Cell Bank, Pasteur Institute of Iran. The MDCK-SIAT1 cell line was kindly provided by Dr Talat Mokhtari-Azad (Tehran University of Medical Sciences, Tehran, Iran). Influenza A/PR8/34(H1N1) was a gift from Xavier Saelens (University of Ghent, Ghent, Belgium).

Transfection and Western blot analysis

MDCK cells were transfected by recombinant pcDNA3.1 (−) containing the Beclin-1 (NM_003766.3) using lipofectamine 3000. Forty-eight hours after transfection, cells were subjected to freeze and thaw cycles three times, followed by clarification using centrifugation. Then, the cell lysates were boiled for 10 min. Protein samples were run in SDS-PAGE gel and the expression level of Beclin-1 was confirmed by Western blot. Since a histidine tag had been added to the 3′ end of the Beclin-1 gene, expression of the gene was confirmed by His-tag-specific antibody. Briefly, the expressed protein was electrophoresed in a 12 % polyacrylamide gel. Next, the protein band was transferred to nitrocellulose membrane (Sartorius) and detected using mouse anti-6×His tag mAb (Abcam) as the primary antibody and peroxidase-conjugated goat anti-mouse immunoglobulin (Dako) as the secondary antibody. Finally, 3, 3′-diaminobenzidine (Amersham Bioscience) was used as chromogenic substrate to visualize the proteins.

Detection of autophagy induction

Induction of autophagy was confirmed by autophagosomal marker LC3-II detection using specific antibodies and flow cytometry. Briefly, at 24 h following the transfection with Beclin-1, the supernatants from confluent cells were removed, and the cells were suspended and fixed in 4 % formaldehyde for 15 min. Cells were then permeabilized using 0.2 % Trixton X-100 for 10 min at room temperature (RT). Finally, the cells were covered with the primary antibody against LC3-II diluted in PBS containing 1 % BSA for 1 h. After washing with PBS, FITC-conjugated secondary antibody was added and incubated at RT for 1 h [15]. The stained cells were examined by flow cytometry (Partec). Cells transfected with pcDNA empty vector were used as the negative control.

Cell transfection and virus inoculation

MDCK and MDCK-SIAT1 cell lines were cultured in DMEM containing 10 % FBS, 100 units penicillin G ml^{-1} and 100 µg streptomycin ml^{-1} in 6-well plates. Cells with 80 % confluency were washed with PBS to remove traces of antibiotics and FBS. In the prophylactic approach, cells were first transfected with pcDNA-Beclin-1 using lipofectamine 3000 with a ratio of 1 : 2. After 1 h incubation at 37 °C, serum-free media containing glutamine was added to each well. Following 4 h incubation, all media were discarded and the virus inoculum was added to the cells (m.o.i. 0.01). Following 1 h incubation for virus adsorption, cells were rinsed with DMEM. Then, media containing 1 % TPCK (tosyl phenylalanyl chloromethyl ketone; Gibco) was added. The cells were incubated for 48 h. In the other procedure (the therapeutic approach), cells were inoculated with the virus first, then transfected with the mix of plasmid and lipofectamine 3000. The virus titre in the two cell lines was measured using a haemagglutination assay and the $TCID_{50}$ (tissue culture infective dose giving 50 % infection) of the viruses was calculated by the Karber formula [16] at 24 and 48 h following inoculation. Virus-inoculated and untreated cells were considered as positive and negative controls, respectively [17, 18].

Haemagglutination assay

Fifty microlitres supernatant from infected cells was harvested 24 and 48 h post-infection. The supernatant was

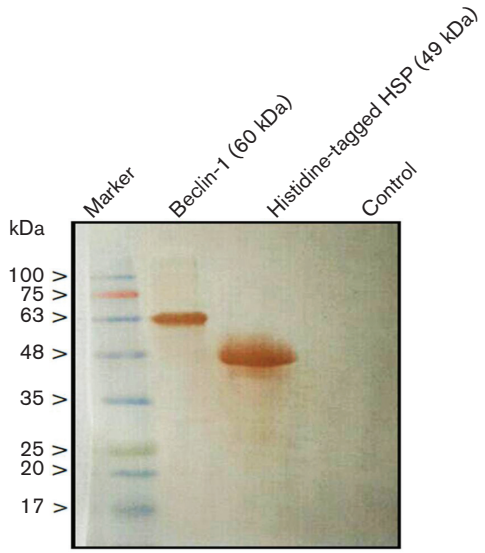

Fig. 1. Western blot analysis of Beclin-1 expression. Forty-eight hours after transfection with Beclin-1, the MDCK cells were washed with PBS and scraped into Tris buffer (pH 8). Lanes: 1, protein marker; 2, Beclin-1 (60 kDa); 3, histidine-tagged heat shock protein (HSP) as a positive control (49 kDa); 4, negative control.

diluted in twofold serial dilutions with PBS and 50 µl chicken red blood cells (1 %) was added to each dilution in a V-shaped microtitre plate. After a gentle agitation, the plates were left undisturbed for 30 min at RT. The last dilution showing complete haemagglutination was considered as the end point and was presented as a haemagglutination unit (HAU) value per test volume [19].

Virus titres in MDCK and MDCK-SIAT cells

All the collected supernatants were 10-fold serially diluted and added to 96-well plates with 80 % confluent cells in triplicate and incubated for 48 h. One hundred microlitres of the supernatants was collected and subjected to $TCID_{50}$ calculation using the Karber formula [17].

3-MA treatment and IV plaque assay

MDCK cells were infected with IAV (m.o.i. of 5.0). After 1 h, the cells were rinsed twice with serum-free medium. Cells were then treated with different concentrations of 3-MA ranging from 0.1 to 10 µM in serum-free medium containing 1 % TPCK/trypsin. Following 24 h, the cells and supernatants were collected and subjected to freeze–thaw cycles three times. Cell suspensions were centrifuged at 4000 r.p.m. for 30 min and the supernatants were collected. In order to perform virus titration, 10-fold serial dilutions of these supernatants were prepared and used to inoculate confluent MDCK cells in 6-well plates. Virus replication was measured using a plaque assay. Cells were overlaid with DMEM containing 0.8 % agarose and TPCK, incubated for 2 days at 37 °C and then overlaid with crystal violet.

RESULTS

Beclin-1 expression

Beclin-1 expression was confirmed by Western blot using mouse anti-6×His tag mAb, as our designed recombinant Beclin-1 had a histidine tag at the C-terminal. As shown in Fig. 1, proteins blotted onto membrane were visualized after peroxidase-based staining with 3, 3′-diaminobenzidine.

Autophagy induction

Staining of LC3-II as an autophagosomal marker showed that in the cells transfected with pcDNA-Beclin-1, the LC3-II level was increased to 16 %. However, the LC3-II level in the cells infected by virus and transfected with pcDNA-Beclin-1 increased to 26 % (Fig. 2).

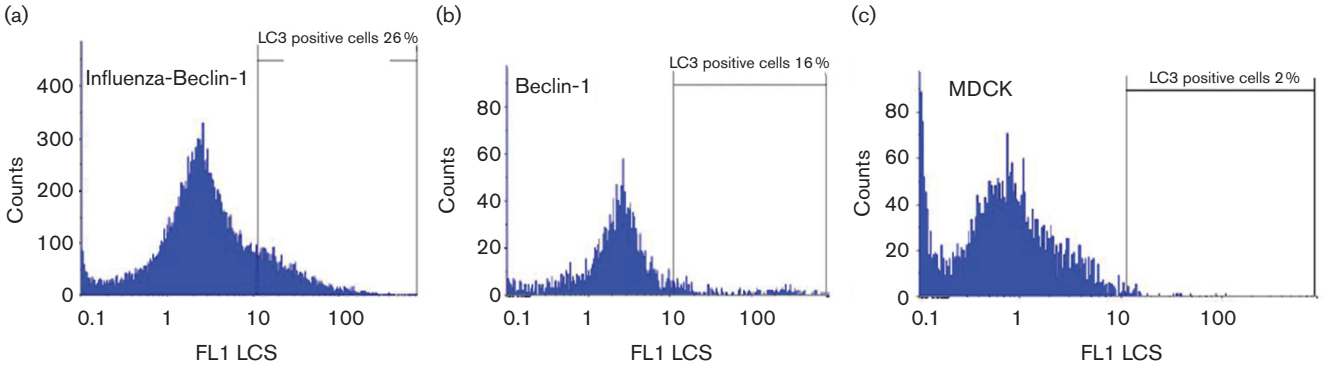

Fig. 2. Autophagy induction. Induction of autophagy was confirmed by the staining of autophagosomal marker LC3-II with specific antibodies and using flow cytometry analysis. The FITC detector FL1 stands for the mean of fluorescence intensities. (a) The LC3-II level in the cells infected by virus and transfected with pcDNA-Beclin-1 was increased to 26 %. (b) The cells transfected with Beclin-1 induced accumulation of LC3-II dots in the phagophore. More than 16 % of the Beclin-1 transfected cells were LC3-II positive. (c) Non-transfected MDCK cells.

Table 1. log_{10} HA and infectivity of IV in MDCK and MDCK-SIAT1 cell supernatants for the prophylactic approach

Values for log_{10} HA are the means of three independent repeats. Values for $TCID_{50}$ are the means of three replicates.

Time post-infection (h)	Cell line used for virus production							
	MDCK				MDCK-SIAT1			
	24		48		24		48	
	log_{10} HA (±SD)	$TCID_{50}$	log_{10} HA (±SD)	$TCID_{50}$	log_{10} HA (±SD)	$TCID_{50}$	log_{10} HA (±SD)	$TCID_{50}$
Virus control	2.71±0.00	10^7	2.31±0.14	$10^{6.5}$	2.31±0.14	$10^{6.5}$	2.71±0.00	10^7
Prophylactic approach†	2.81±0.28	$10^{8.5}$**	2.31±0.14	$10^{6.5}$	2.91±0.14**	$10^{8.5}$**	2.61±0.14	10^7

†Two asterisks indicate a highly significant difference ($P \leq 0.01$).

Haemagglutination and virus infectivity assay

The results of the haemagglutination assay and virus infectivity are shown in Tables 1 and 2. The log_{10} haemagglutinin (HA) titres and virus infectivity ($TCID_{50}$) did show significant differences at 24 h post-infection, but not at 48 h. As shown in the Table 1, during 24 h exposure, in the prophylactic approach, virus titre and infectivity increased in both cell lines, but for the same exposure method at 48 h, virus titre and infectivity dropped in both cell lines to the level of the virus infection control. However, in the therapeutic approach (shown in Table 2), the autophagy induction in both cell lines inhibited virus titre and infectivity ($P \leq 0.01$).

Inhibition of autophagy

To test the effect of autophagy inhibition on virus growth, we infected MDCK cells with IAV and treated the infected cells in a dose-response manner with 3-MA, an autophagy inhibitor (Fig. 3). Examining the virus titre in treated cells indicated that 3-MA reduced viral replication at the concentration of 10 μM by about 70 %, suggesting that inhibition of autophagy affects IAV replication.

DISCUSSION

Interactions between viruses and the autophagy pathway have determining effects on the fate of the virus replication, which can have antiviral or pro-viral forms. IAV stimulates the formation of autophagosomes in favour of the virus life cycle, escaping from the immune defences and activating autophagic cell death. Conversely, IAV blocks the maturation of autophagy and inhibits the digestion of autophagic contents by lysosomes; thus, resulting in the augmentation of viral elements and causing apoptotic cell death [13].

Time-course monitoring of IV replication after autophagy induction and suppression has not been well addressed. To this end, we evaluated the effect of autophagy induction on IAV replication. Beclin-1 was expressed in MDCK and MDCK-SIAT1 cells, before and after viral infection. In the prophylactic approach, where Beclin-1 was expressed 24 h before infection, virus titre and infectivity increased at 24 h post-infection, but reduced at 48 h post-infection in both cell lines. In parallel with the current study, Guévin *et al.* [20] have reported that the interaction between hepatitis C virus and the autophagy system is regulated in a temporal manner. Furthermore, Dreux and colleagues from the Chisari lab emphasized the involvement of the autophagy machinery in the initiation of virus replication, while it becomes dispensable for hepatitis C virus progeny production as soon as replication is established [10].

In the therapeutic approach, at 24 and 48 h in both of the cell lines, virus titre and infectivity decreased ($P \leq 0.01$). In accord with this, Liang *et al.* reported that Beclin-1

Table 2. log_{10} HA and infectivity of IV in MDCK and MDCK-SIAT1 cell supernatants for the therapeutic approach

Values for log_{10} HA are the means of three independent repeats. Values for $TCID_{50}$ are the means of three replicates.

Time post-infection (h)	Cell line used for virus production							
	MDCK				MDCK-SIAT1			
	24		48		24		48	
	log_{10} HA (±SD)	$TCID_{50}$	log_{10} HA (±SD)	$TCID_{50}$	log_{10} HA (±SD)	$TCID_{50}$	log_{10} HA (±SD)	$TCID_{50}$
Virus control	2.71±0.00	10^7	2.31±0.14	$10^{6.5}$	2.31±0.14	$10^{6.5}$	2.71±0.00	10^7
Therapeutic approach†	2.01±0.14**	10^6**	2.01±0.14*	10^6*	2.01±0.14*	10^6*	2.01±0.14**	10^6**

†One asterisk indicates a significant difference ($P \leq 0.05$) and two asterisks indicate a highly significant difference ($P \leq 0.01$).

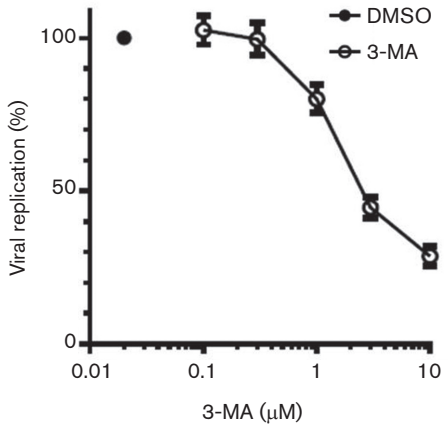

Fig. 3. Inhibition of autophagy reduces IV replication. Different concentrations of 3-MA, ranging from 0.1 to 10 μM, were added to MDCK cells infected with IV at an m.o.i. of 5.0. Virus replication was measured using a plaque assay.

expression from a recombinant Sindbis virus could decrease virus titre and protect mice from fatal encephalitis [21]. Exposing infected cells to 3-MA at a concentration of 10 μM reduces the virus yield by approximately 70 %, and inhibition of autophagy by 3-MA and wortmannin decreases the IAV titre [11, 22].

The result of virus inoculation before transfection with Beclin-1 indicated the key role of autophagosome induction against viral replication, but inhibition of the autophagosome showed a significant reduction in the IV yield. It has been reported that infection by IAV enhances the formation of autophagosomes in mammalian cells [11]. M2 protein alone is sufficient to induce the initial steps of autophagosome formation [11, 12, 23], but M2 contains an LC3-interacting domain, which is essential to prevent autophagosome maturation [12, 24]. M2 also binds to Beclin-1, which could trigger autophagy inhibition through disrupting the Beclin-1 complex [6]. Gannagé *et al.* [12] showed that M2 protein silencing in IV leads to fusion of the autophagosome with lysosome and degradation of the virus. All these processes might be mediated through the interaction with Beclin-1 [6, 12]. Furthermore, only live IV is capable of inducing autophagosome formation, but not weakened or dead viruses [25].

Based on the results of the prophylactic approach, we hypothesize that overexpression of Beclin-1 causes M2 proteins to interact and inhibit the fusion of autophagosomes with lysosomes, resulting in an increase in viral titre. However, in the therapeutic approach, the lack of Beclin-1 causes the inability of M2 proteins to control autophagosome fusion with lysosome, resulting in a decreased viral titre. Future studies will address the cross-talk between IV and autophagy during the virus life cycle.

Taken together, little is understood about how the autophagy pathway interacts with viruses. Elucidating this mutual interaction provides a promising field for the design of new antiviral compounds and novel virus replication inducers.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This article does not contain any studies with human participants or animals performed by any of the authors.

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