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 ≤ 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...
inhibit autophagosome formation to escape autophagic degra-
dation [6]. However, human immunodeficiency virus 1, polio-
virus 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 autophago-
some formation through interaction with signalling pathways
and autophagy-related genes. In addition, it was reported that IAV-induced apoptotic cell deaths were signifi-
cantly enhanced in autophagy-deficient cells [12]. Interest-
ingly, highly pathogenic strains of IAV, such as H5N1,
induce autophagic cell death and interact with the autoph-
agy machinery using mechanisms different from those util-
lized by weakly pathogenic strains [13, 14].

In this study, we analysed the effects on virus titre of target-
ing 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 inocula-
tion. 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-meth-
yladenine (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 Apcam. 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 elec-
trophoresed 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 % Tixon X-100 for 10 min at room temperature
(RT). Finally, the cells were covered with the primary anti-
body 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 lipofect-
amine 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 dis-
carded 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 proce-
dure (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 TCID₅₀
(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 har-
vested 24 and 48 h post-infection. The supernatant was
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\textsubscript{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).
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≤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≤0.01). In accord with this, Liang et al. reported that Beclin-1

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

<table>
<thead>
<tr>
<th>Time post-infection (h)</th>
<th>Cell line used for virus production</th>
<th>MDCK</th>
<th></th>
<th>MDCK-SIAT1</th>
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<td></td>
<td></td>
<td>24</td>
<td>48</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>log_{10} HA (±SD)</td>
<td>TCID_{50}</td>
<td>log_{10} HA (±SD)</td>
<td>TCID_{50}</td>
</tr>
<tr>
<td>Virus control</td>
<td></td>
<td>2.71±0.00</td>
<td>10^7</td>
<td>2.31±0.14</td>
<td>10^6.5</td>
</tr>
<tr>
<td>Prophylactic approach†</td>
<td></td>
<td>2.81±0.28</td>
<td>10^6**</td>
<td>2.31±0.14</td>
<td>10^6.5</td>
</tr>
</tbody>
</table>

†Two asterisks indicate a highly significant difference (P≤0.01).

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

<table>
<thead>
<tr>
<th>Time post-infection (h)</th>
<th>Cell line used for virus production</th>
<th>MDCK</th>
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<th>MDCK-SIAT1</th>
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<td>2.31±0.14</td>
<td>10^6.5</td>
</tr>
<tr>
<td>Therapeutic approach†</td>
<td></td>
<td>2.01±0.14**</td>
<td>10^6**</td>
<td>2.01±0.14*</td>
<td>10^6*</td>
</tr>
</tbody>
</table>

†One asterisk indicates a significant difference (P≤0.05) and two asterisks indicate a highly significant difference (P≤0.01).
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 crosstalk 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.

**References**


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