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ORIGINAL ARTICLE

Beclin 1 is Involved in Regulation of Apoptosis and Autophagy During Replication of Ectromelia Virus in Permissive L929 Cells

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Abstract Several reports have brought to light new and interesting findings on the involvement of autophagy and apoptosis in pathogenesis of viral and bacterial diseases, as well as presentation of foreign antigens. Our model studies focused on the involvement of apoptosis during replication of highly virulent Moscow strain of ectromelia virus (ECTV-MOS). Here, we show evidence that autophagy is induced during mousepox replication in a cell line. Fluorescence microscopy revealed increase of LC3 (microtubule-associated protein 1 light chain 3) aggregation in infected as opposed to non-infected control L929 cells. Furthermore, Western blot analysis showed that replication of ECTV-MOS in L929 cells led to the increase in LC3-II (marker of autophagic activity) expression. Beclin 1 strongly colocalized with extranuclear viral replication centers in infected cells, whereas expression of Bcl-2 decreased in those centers as shown by fluorescence microscopy. Loss of Beclin 1-Bcl-2 interaction may lead to autophagy in virus-infected L929 cells. To assess if Beclin 1 has a role in regulation of apoptosis during ECTV-MOS infection, we used small interfering RNA directed against *beclin 1* following infection. Early and late apoptotic cells were analyzed by flow cytometry after AnnexinV and propidium iodide staining. Silencing of *beclin 1* resulted in decreased percentage of early and late apoptotic cells in the late stage of ECTV-MOS infection in L929 cells. We conclude that Beclin 1 plays an important role in regulation of both, autophagy and apoptosis, during ECTV-MOS replication in L929 permissive cells.

Keywords Ectromelia (mousepox) virus · Autophagy · Apoptosis

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Introduction

Ectromelia virus (ECTV; mousepox ["smallpox of mice"] virus) is a large (220–450 nm long and 140–260 wide) double stranded-DNA virus which causes a generalized infection and as such is an excellent model for studying viral pathogenesis (Boratyńska et al. 2010; Cespedes et al. 2001; Krzyżowska et al. 2002, 2005, 2006; Szulc et al. 2010). ECTV belongs to the family *Poxviridae*, genus *Orthopoxvirus* together with variola virus (the causative agent of smallpox responsible for millions of death in the history of mankind), cowpox virus, vaccinia virus (VACV), and other orthopoxviruses closely related to ECTV (Esteban and Buller 2005). Our earlier studies (Krzyżowska et al. 2002) showed that DEVD-ase-dependent (caspase-3 and caspase-7) apoptosis (type I cell death) is an important mechanism regulating the resolution of ECTV-MOS infection in L929 and RK-13 cell lines and played an important role in pathogenesis of ECTV. In the present study, we have asked whether autophagy (type II cell death) is also involved in the pathogenesis of ECTV.

Autophagy plays an important role in microbial pathogenesis, particularly it promotes killing of intracellular microorganisms by delivering them to degradative lysosomes (a process known as xenophagy). For instance *Toxoplasma gonidii*, group A *Streptococcus, Mycobacterium tubercuolosis, Shigella flexneri, Salmonella enterica, Listeria monocytogenes, Francisella tularensis* and herpes

simplex virus type I (HSV-1) have been reported to undergo xenophagy (Levine and Kroemer 2008). Physiological or pharmacological stimulation of autophagy in macrophages suppresses intracellular survival of mycobacteria by promoting the maturation of phagosomes into phagolysosomes (Deretic et al. 2006; Gutierrez et al. 2004). However, some viruses e.g. HSV-1 can inhibit autophagy through ICP34.5 protein (infected cell protein 34.5) synthesis (Orvedahl et al. 2007). On the other hand, autophagy may contribute to survival of bacteria such as *Coxiella burnetii* (Colombo et al. 2006) or facilitate replication of poliovirus (Jackson et al. 2005). It is also known that autophagy may lead to apoptosis of bystander $CD4^+$ T lymphocytes in human immunodeficiency virus type 1 (HIV-1) infection (Espert et al. 2006, 2007).

Autophagy is usually divided into three types: macroautophagy (MA), microautophagy, and chaperone mediated autophagy (CMA) (Kaushik and Cuervo 2006; Majeski and Dice 2004; Shintani and Klionsky 2004). MA, often called autophagy, is the process in which a double membrane structure called autophagosome sequesters targets substrates such as organelles, pathogens, and macromolecules and delivers them to lysosomes. This is the best characterized type of autophagy regulated by at least 27 autophagy related (Atg) genes, involving two ubiquitin-like conjugation systems: Atg12-Atg5 and Atg8-phosphatidylethanolamine (PE). In mammalian cells MAP1-LC3 (microtubule-associated protein 1 light chain 3, LC3) is one of the homologues of yeast Atg8 and a 16 kDa isoform of LC3—LC3-II, corresponds to Atg8-PE in yeast (Geng and Klionsky 2008). MA is also regulated by: (i) class III (upregulates), (ii) class I 14phosphoinositide 3-kinase and (iii) mTOR (mammalian target of rapamycine) kinase which is a specific cell starvation sensor inhibiting this process (Klionsky and Emr 2000; Klionsky 2005). After fusion of the autophagosome with lysosome its inner membrane is digested. CMA occurs when a portion of the cytoplasm is engulfed by a whole lysosome through invagination of the lysosomal membrane. Substrate proteins for chaperone mediated autophagy contain the pentapeptide motif KFERQ. This type of autophagy is dependent on cytoplasmatic heat shock cognate protein of 73 kDa—Hsc70, which recognizes KFERQ motif of targeted proteins. CMA substrate degradation/synthesis requires interaction of lysosomal associated membrane protein-2 with targeted protein although translocation is dependent on the presence of lysosomal Hsc70 (Mizushima et al. 2002, 2003).

Despite the marked differences between autophagy and apoptosis, regulation of both processes is intimately connected and requires similar molecular regulatory proteins. For that reason, Thorburn (2008) suggested that these processes are different facets of the same cell death. An example of molecular connection between apoptosis and autophagy is anti-apoptotic protein: B-cell lymphoma 2 (Bcl-2). Bcl-2 can interact with Beclin 1 (Bcl-2 interacting protein-1)/Atg6, which is responsible for formation of the autophagic vesicle. Bcl-2 interference with Beclin 1 prevents autophagy induction dependent on Beclin 1 (Pattingre et al. 2005; Thorburn 2008) (Fig. 1).

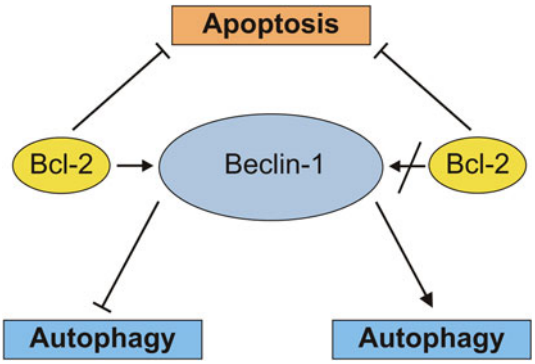

Fig. 1 Apoptosis (type I cell death) and autophagy (type II cell death) relationship based on Beclin 1–Bcl-2 interactions

In this paper, we present evidence of type II cell death (autophagy) involvement in in vitro replication of highly infectious Moscow strain of ECTV (ECTV-MOS) in permissive L929 cells. Our results show high levels of LC3-II protein which can be attributed to autophagic activity.

Materials and Methods

Virus

In this study, ECTV-MOS (ATCC 1374) propagated in permissive Vero cells (ATCC CCL-81, African green monkey kidney epithelial adherent cells) was used in all experiments described. Infectivity of the virus was determined by plaque assay on Vero cells according to standard procedures. Virus was stored at −70°C until use in experiments.

Cell Culture

Murine fibroblasts (clone L-929, ATCC number CCL-1, derived from C3H/An mouse) were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 5% fetal bovine serum (FBS; Invitrogen, USA). All cell lines were incubated at 37°C in a 4.5% CO_2 humidified atmosphere. Infection of L929 cells with ECTV was carried out at the multiplicity of infection (MOI) 5 plaque forming units (PFU)/cell in DMEM supplemented with 1% FBS or bovine calf serum (Sigma, USA) and

antibiotic–antimycotic (100 U/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amfotericin) (Sigma, USA).

Reagents

Autophagy was analyzed using the following antibodies (Abs): rabbit anti-LC3, rabbit anti-Beclin 1 and secondary antibodies conjugated with FITC against rabbit Abs (all were purchased from Sigma, USA). Bcl-2 was detected with Armenian hamster anti-Bcl-2 (BD Bioscience, USA) and goat anti-Armenian hamster Ig conjugated to rodamine-RedX (Jackson ImmunoResearch Laboratories, USA) Abs. Rabbit polyclonal anti-ECTV-MOS (pAb) conjugated to FITC was used to detect virus as proof of infection, as previously described (Boratyńska et al. 2010). In experiments involving Western blot HRP-conjugated anti-rabbit secondary antibodies (Sigma, USA) were used. Intracellular staining was performed using Cytofix/Cytoperm and Perm/Wash purchased from BD Bioscience. Beclin 1 small interfering (si)RNA transfection kit was used to knockdown gene expression (Santa Cruz Biotechnology, USA). The percentage of apoptotic cells was determined with Annexin V-FITC Apoptosis Detection Kit (Sigma, USA).

siRNA Transfection

Expression of Beclin 1 in L929 cell was inhibited by BECN1 siRNA (Santa Cruz Biotechnology, USA). Briefly, cells were grown in 6-well tissue culture slides in DMEM medium supplemented with 5% FBS without antibiotic/antimycotic. Shortly before transfection cells were washed with transfection medium and then *Beclin 1* siRNA (60 pmol/100 μl) prepared in siRNA Transfection Reagent (Santa Cruz Biotechnology, USA) was added according to the manufacturer's instructions. Cells were infected with ECTV-MOS 24 h after transfection and collected at particular time points for immunoblot or flow cytometric analysis.

Electrophoresis and Western Blotting

Analysis of Beclin 1 and LC3 expression was performed at 2, 4, 6, 12, 18 and 24 h post infection (h.p.i.) of L929 cells with ECTV-MOS. Whole cell lysates were prepared using Mammalian Cell Lysis Kit (MCL1; Sigma, USA), then the protein content was measured by bicinchoninic acid (BCA) assay (QuantiPRO BCA Assay Kit; Sigma, USA) in accordance with the manufacturer's instructions. Electrophoresis of Beclin 1 (60 kDa) and LC3 (16 kDa) proteins was conducted on 12 and 15% polyacrylamide gels, respectively. Protein blotting on polyvinylidene fluoride membrane was run at 70 V for 60 min. Chemiluminescence was detected on X-Ray film Kodak Retina using Chemiluminescent Peroxidase Substrate-1 (Sigma, USA) according to the manufacturer's manual. Western blot images were taken on KODAK Image Station 4000 MM digital imaging system and data were analyzed using KODAK MI SE software.

Fluorescence Microscopy

Cells were cultured in 6-well tissue culture slides on coverslips to 70% confluence and infected with ECTV at MOI of 5. Later at 18 h.p.i. cells were fixed and permeabilized with BD Cytofix/Cytoperm for 25 min on ice. Then, cells were incubated for 1 h on ice with primary antibodies against appropriate markers diluted at 1/200. Next, the cells were washed in Perm/Wash solution and stained for 60 min on ice with appropriate secondary antibodies diluted at 1/500. The nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI; Vector Lab., USA). Images were recorded using fluorescence microscope (Olympus BX60, Japan) equipped with Color View III cooled CCD camera and Cell^F software (Olympus, Japan).

Flow Cytometry

Beclin 1 siRNA-transfected and non-transfected L929 cells at 18 h.p.i. with ECTV-MOS were stained with Annexin V-FITC Apoptosis Detection Kit (Sigma, USA) according to the manufacturer's protocol. The percentage of apoptotic cells was determined by flow cytometric analysis. 10,000 events were acquired in FACSCalibur flow cytometer with CellQuest software (Becton–Dickinson, USA).

Statistical Analysis

All quantitative data are expressed as mean ± SD from at least three independent experiments. Western Blotting experiments were analyzed using Mann–Whitney *U* test, and Student's *t* test was used for flow cytometric analysis. Significance was assessed at $p \leq 0.05$. All analyses were performed with Statistica 6.0 software (Statsoft Inc., USA).

Results

Expression of LC3-II and Beclin 1 Proteins

Expression of LC3 protein in control and ECTV-MOS infected L929 cells was initially evaluated by fluorescence microscopy. Uninfected L929 cells displayed a diffuse cytoplasmic localization of LC3 protein (Fig. 2a), whereas ECTV-MOS-infected L929 cells showed an increase in

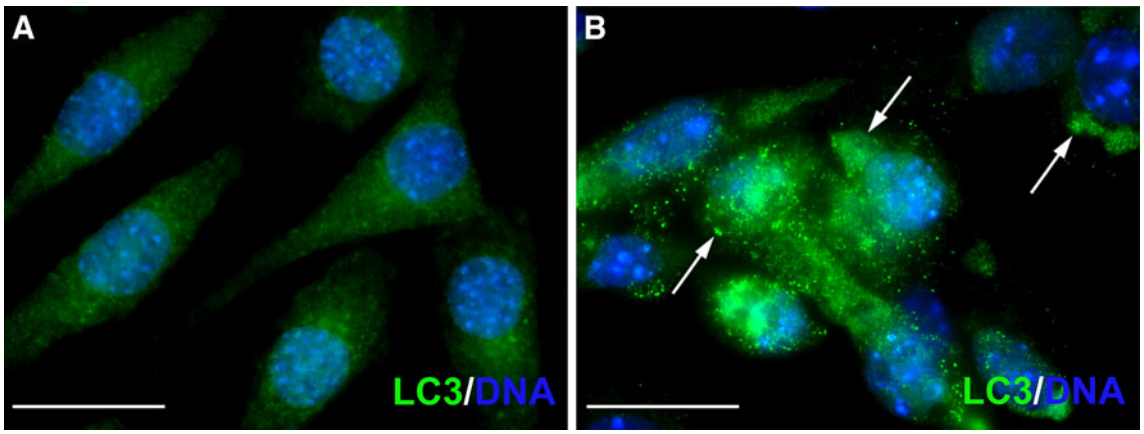

Fig. 2 Distribution of LC3 protein in uninfected (**a**) and infected L929 cells at 18 h.p.i. with ECTV-MOS (**b**). *White arrows* indicate aggregated LC3 protein. *Scale bars* 30 μm

punctate aggregates of LC3 protein within the cytoplasm (Fig. 2b). This result is consistent with the autophagosome formation (Klionsky et al. 2008).

Microscopy analysis of LC3-II protein expression using available antibodies does not allow to distinguish between two forms of LC3 protein: LC3-II (16 kDa) and its 18 kDa LC3-I form (Asanuma et al. 2003). Meanwhile, the amount of LC3-II correlates with the number of autophagosomes and LC3-II is an autophagosomal marker. For that reason we additionally assessed LC3-II expression by Western blot analysis, which allows to distinguish between the two forms of LC3 protein.

Representative analysis of LC3-II (a 16 kDa isoform of LC3) protein levels during ECTV-MOS infection of L929 cells is presented in Fig. 3a. LC3-II expression increased significantly ($p \leq 0.05$) as early as 4 h.p.i. and between 18 and 24 h.p.i. the protein level in infected L929 cell was twofold higher compared to uninfected control cells (2.18 and 1, respectively) (Fig. 4). Therefore, the increase in LC3-II expression was observed in almost all stages of ECTV-MOS replication cycle.

Western blot analysis of Beclin 1 (60 kDa) did not reveal any changes in its expression level during ECTV-MOS replication cycle in L929 cells (Fig. 3b). However, intracellular distribution of Beclin 1 in control and infected L929 cells varied as determined by fluorescence microscopy (Fig. 5). Noninfected L929 cells displayed diffuse localization of Beclin 1 within the cytoplasm (Fig. 5a). Meanwhile, in L929 cells at 18 h.p.i. Beclin 1 strongly colocalized with the extranuclear virus replication centers (Fig. 5b). Those centers stained intensively with pAb anti-ECTV-MOS (Fig. 6). Such distribution of Beclin 1 suggests its involvement in ECTV-MOS replication; however, this statement should be confirmed in further studies. Moreover, it is known that this phylogenetically conserved protein is essential for the initiation of autophagy, perhaps

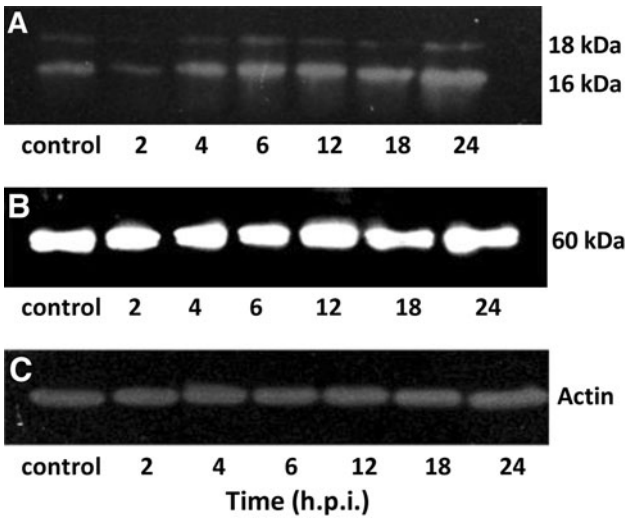

Fig. 3 Representative Western blot analysis of LC3-I and LC3-II (**a**, 18 and 16 kDa, respectively) Beclin-1 (60 kDa; **b**) and *β*-actin (**c**) expression in uninfected (control) and infected L929 cells at 2, 4, 6, 12, 18 and 24 h.p.i. with ECTV-MOS

via its interaction with class III phosphatidylinositol-3-kinase Vps34 (Zeng et al. 2006).

Bcl-2-Beclin 1 Interactions

Bcl-2 and Beclin 1 physically interact through the BH3 domain within Beclin 1 (residues 114–123) (Maiuri et al. 2007). We asked if this interaction occurred during infection with ECTV. Studies based on immunofluorescence microscopy analysis revealed colocalization of Bcl-2 and Beclin 1 proteins within the cytoplasm of uninfected L929 cells (Fig. 5a). Meanwhile, in L929 cells at 18 h.p.i with ECTV-MOS there was a decrease in Bcl-2–Beclin 1 colocalization in the cytoplasm, especially within the sites of virus replication (Fig. 5b), mainly due to reduction in

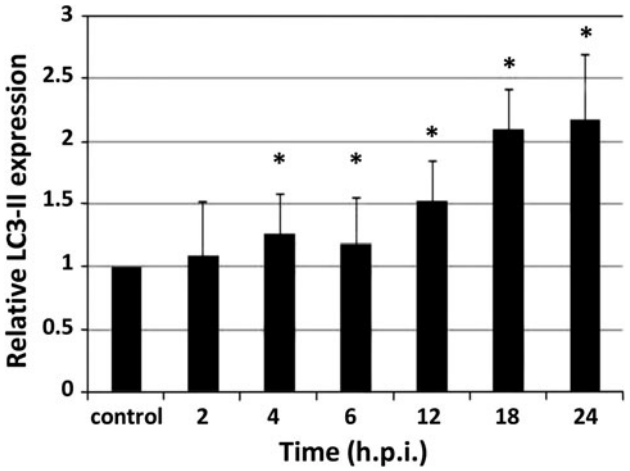

Fig. 4 LC3-II (16 kDa) expression in uninfected (control) and infected L929 cells at 2, 4, 6, 12, 18 and 24 h.p.i. with ECTV-MOS. Data are shown as a multiplicity of uninfected (control) protein level. Data are expressed as mean ± SD values from three independent experiments. Significant differences in LC3-II levels between control and ECTV-MOS-infected cells were demonstrated by Mann–Whitney *U* test ($*p \leq 0.05$)

the expression of Bcl-2. The decrease of Bcl-2-Beclin 1 interactions in infected L929 cells may drive those cells to autophagy during replication of ECTV-MOS; however, usage of other anti-Bcl-2 antibodies may be required to confirm this statement.

Beclin 1 Regulates Apoptosis During ECTV-MOS Replication

To confirm a direct role of Beclin 1 in ECTV-MOS induced cell death, we used siRNA to specifically inhibit Beclin 1 expression. Apoptosis was quantified by Annexin V-FITC, which binds to phosphatidylserine residues that are translocated from the inner face of the plasma membrane to the cell surface during early events in apoptosis. Propidium iodide (PI) cannot penetrate the intact cell membrane, but after the loss of membrane integrity, PI can enter the cell and intercalate into DNA. Viable cells are Annexin V^-/PI^-. The Annexin V^+/PI^- and Annexin V^+/PI^+ are early and late apoptotic cells, respectively (Fig. 7b). Experiments were performed only on *beclin 1*

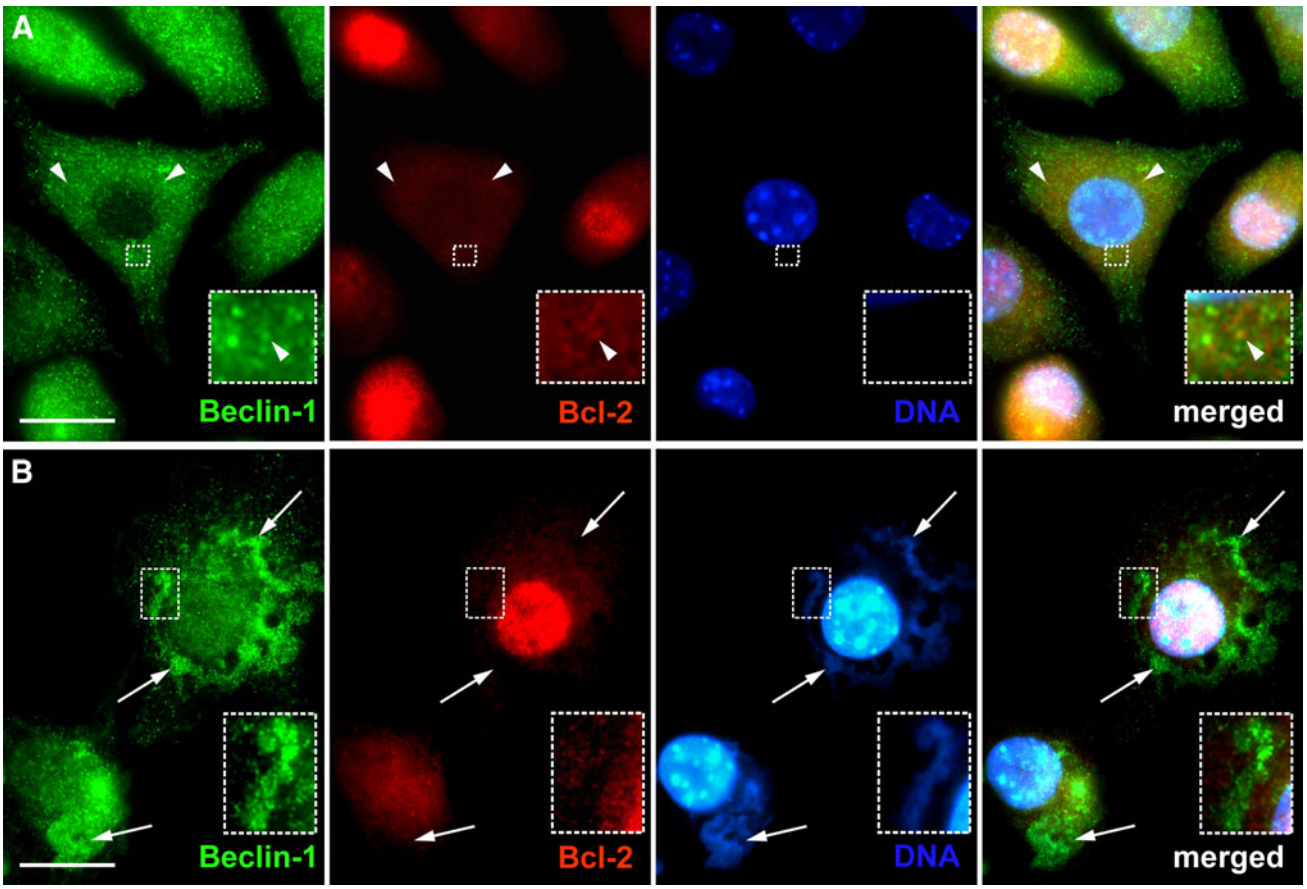

Fig. 5 Microscopy analysis of Beclin 1 (*green*), Bcl-2 (*red*) and cellular nuclei and viral DNA (*blue*) distribution in L929 cells uninfected (**a**) and infected with ECTV-MOS at 18 h.p.i. (**b**). *White arrowheads* show colocalization of Beclin 1 with Bcl-2 in control cells (**a**), *white arrows* indicate colocalization of viral DNA with Beclin 1 (**b**). *Scale bars* 20 μm

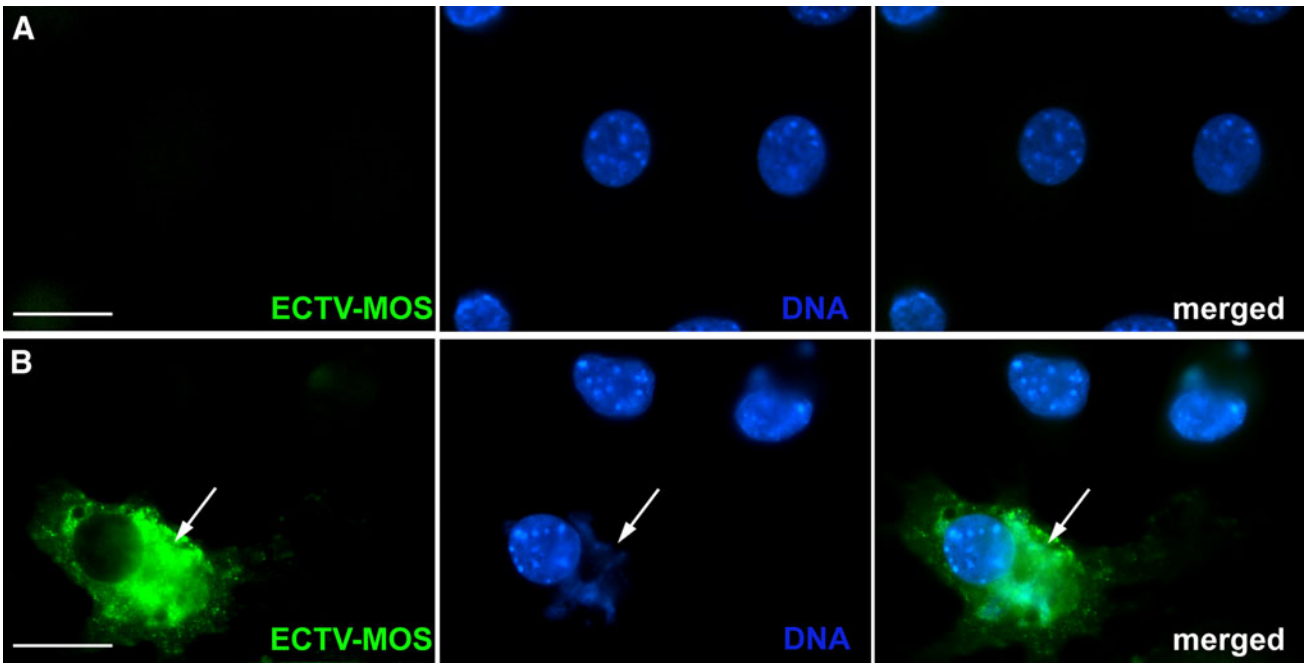

Fig. 6 Distribution of ECTV-MOS antigens in infected L929 cells. Uninfected L929 cells stained against ECTV-MOS are shown as a specificity control (**a**). Cells at 18 h.p.i. with ECTV-MOS show colocalization of viral DNA (*blue*) with ECTV-MOS antigens (*green*) indicated by *arrows* (**b**). *Scale bars* 20 μm

siRNA-transfected L929 cells and *beclin 1* silencing was confirmed by Western blotting analysis (Fig. 7a).

Analysis showed a statistically significant increase ($p \leq 0.01$) of the percentage of late apoptotic cells in L929 cells at 18 h.p.i. with ECTV-MOS compared to uninfected control fibroblasts (11.43 and 3.78%, respectively) (Fig. 7b, c). Meanwhile, at 18 h.p.i. with ECTV-MOS a statistically significant ($p = 0.0242$) decrease in the percentage of Annexin V^+/PI^+ cells was observed in *beclin 1* siRNA-transfected L929 cells compared to non-siRNA-transfected fibroblasts (4.01 vs. 11.43%). Similar differences were observed regarding early apoptosis in both cases (Fig. 7b). This result suggests the involvement of Beclin 1 in apoptosis regulation during the late stages of ECTV-MOS replication in L929 cells.

Discussion

Analysis of autophagy under in vivo conditions is predominantly based on transmission electron microscopy, which allows the differentiation of early and late autophagosomal structures (Martinet et al. 2006). In our experiments, we have studied autophagy in an in vitro model, which exploits L929 fibroblasts, permissive to ECTV-MOS replication.

The data reported in this paper indicate the increase in LC3-II protein level in L929 cell line during all stages of ECTV-MOS replication (4–24 h.p.i.) which is associated with elevated autophagic activity. However, it should be noted that autophagy takes place in numerous cellular processes and as such a precise role for autophagy in ECTV-MOS replication should be defined. One suggestion is that autophagy may be required for effective replication of viral particles. Indeed, some viruses, in particular many positive-stranded RNA viruses, require components of the autophagic machinery for robust replication (Jackson et al. 2005; Martyniszyn et al. 2008). Members of *Picornaviridae* (e.g. poliovirus and coxsackieviruses B3 and B4) and *Flaviviridae* (HCV, hepatitis C virus and DENV, dengue virus) families have been shown to require autophagy for efficient replication (Heaton and Randall 2010). Autophagy is also important in delivery and turnover of amino acids and nutrient components during starvation, this relates to viral replication period when cellular synthesis is inhibited and many components are required for proper coupling of viral particles. It has also been reported that in several cases autophagosomal vesicles may serve as a niche for pathogen survival (Ogawa and Sasakawa 2006). On the other hand, elevated autophagosomal activity is a way of cellular defense against intracellular pathogens. Autophagy (xenophagy) is used to degrade not only bacterial pathogens that invade intracellularly, but also some mature or newly synthesized viral particles (e.g. HSV-1) that are trapped by autophagosomes and undergo lysosomal degradation (Tallóczy et al. 2006). Moreover, autophagy delivers peptides into the MHC class II antigen presentation pathway thus promoting antigen recognition and

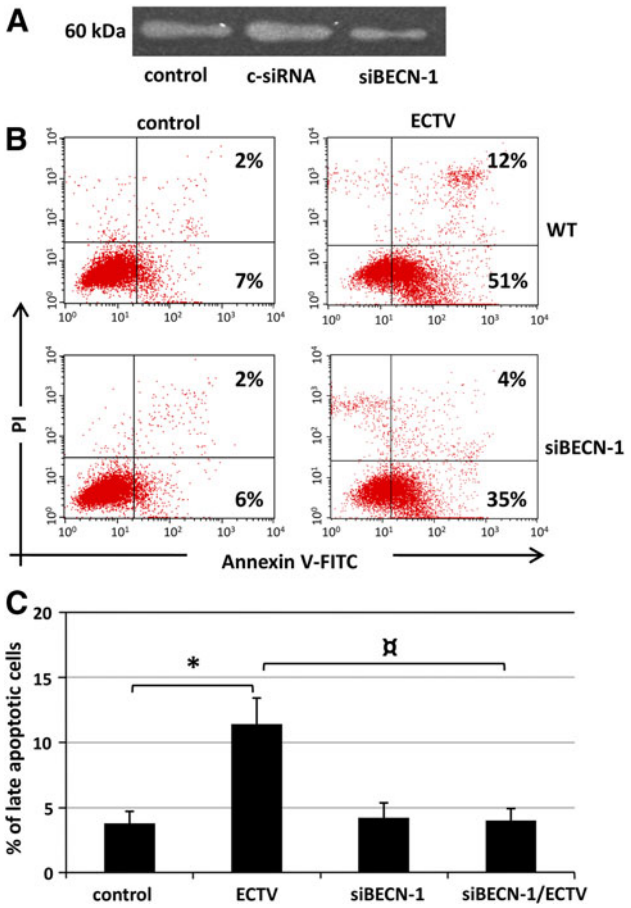

Fig. 7 Flow cytometric analysis of late apoptotic cell percentage in L929 cells and *beclin 1* siRNA-transfected L929 cells at 18 h.p.i. with ECTV-MOS: **a** Western blot analysis of Beclin 1 expression after siRNA (siBECN-1) treatment, compared to untreated control and interference specifity control (c-siRNA); **b** representative dot blots from one of three independent experiments showing the percentage of early (Annexin V^+/PI^-) and late (Annexin V^+/PI^+) apoptotic cells; **c** statistical evaluation of the percentage of late apoptotic cells in uninfected and infected wild-type (WT) L929 cells (control and ECTV, respectively) and in uninfected and infected L929 cells after treatment with siRNA for *beclin 1* transcript (siBECN-1 and siBECN-1/ECTV, respectively). Cells were transfected with siRNA for *beclin 1* (siBECN-1), 24-h post transfection cells were infected with ECTV-MOS (ECTV) and at 18 h.p.i. quantified by flow cytometry after AnnexinV/FITC-PI staining. The values were presented as the mean ± SD from three independent experiments. The differences: ECTV vs control (*asterisk* indicates increase) and siBECN-1/ECTV vs ECTV (*currency sign* indicates decrease) were demonstrated by Student's *t* test ($p \leq 0.05$)

induction of antiviral adaptive immune response (Menéndez-Benito and Neefjes 2007).

Previously, Krzyżowska et al. (2002) showed in vitro involvement of apoptotic cell death of L929 cells in ECTV-MOS infection, consistent with our present observations. These results may suggest a relationship between autophagy and apoptotic cell death during ECTV-MOS replication. Furthermore, autophagy may be involved in proapoptotic alteration through formation of apoptotic bodies, alternatively, it may be a way of avoiding apoptosis or manifestation of normal activity of phagocytes.

Together with LC3-II, Beclin 1 is strongly involved in autophagic process, especially in its early stages (Luo and Rubinsztein 2007). However, our studies did not reveal any changes in the expression level of Beclin 1 during ECTV-MOS replication in L929 cells. Moreover, there was no detectable interaction between Beclin 1 and Bcl-2 proteins in infected fibroblasts, especially within the sites of intensive virus replication, where an increase in Beclin 1 expression with a decrease of Bcl-2 expression was observed. This observation suggests that during the later stages of ECTV-MOS infection Beclin 1 accumulates at the sites of replication and prevents Bcl-2–Beclin 1 interactions, driving the infected cell to autophagy. Moreover, we cannot exclude that such distribution of Beclin 1 may have an important influence on ECTV-MOS replication and/or survival in permissive L929 cells. Same infectious agents can exploit autophagy to enhance their replication (Jackson et al. 2005). Prentice et al. (2004) demonstrated that autophagy is required for efficient replication of mouse hepatitis virus through the formation of intracellular double-membraned vesicles, in which the virus replicates. Other viruses do not require cellular autophagy machinery for their replication cycle (e.g. VACV) (Zhang et al. 2006). It has been reported that Beclin 1 reduces Sindbis virus replication, prevents the death of Sindbis virus-infected neuronal cells and protects mice against lethal Sindbis virus encephalitis (Liang et al. 1998). Some viruses can interact with Beclin 1 leading to inhibition of autophagy. Orvedahl et al. (2007) have shown that HSV-1 neurovirulence protein ICP34.5 antagonizes the autophagy function of Beclin 1. HIV-1 Nef protein interacts with Beclin 1 and acts as an anti-autophagic maturation factor (Kyei et al. 2009). The importance of Beclin 1 distribution to ECTV-MOS replication centers in permissive L929 cells should be determined in future investigations.

Our studies revealed that Bcl-2 expression in uninfected and infected L929 cells may also be nuclear, which is unusual. Recently, Bcl-2 translocation to nuclei has been observed in rat neurons within 24 h of reperfusion after neonatal cerebral hypoxia–ischemia, suggesting the involvement of Bcl-2 in mechanisms other than those based on direct interaction with Bax (Zhu et al. 2010). Moreover, in aged rats there was an oxidative stress-dependent Bcl-2 up-regulation within specialized nuclear compartment; however, nuclear Bcl-2 localization failed to protect cells from apoptosis induced by oxidative stress (Kaufmann et al. 2003). The presence of Bcl-2 in the nucleus suppressed activity of multiple transcription factors including NF-κB through alteration of nuclear trafficking (Massaad et al. 2004). It has been suggested that nuclear localization of Bcl-2 might induce rather than

protect cells from apoptosis and the movement of Bcl-2 was dependent on the interaction between the Bcl-2 BH4 domain and the mitochondrial chaperone protein FKBP38 (Portier and Taglialatela 2006). However, we will re-evaluate Bcl-2 expression using other anti-Bcl-2 antibodies to confirm our statement.

To learn if Beclin 1 has a role in regulation of apoptosis during ECTV-MOS replication in L929 cells, we silenced the *beclin 1* gene within cells followed by infection with the virus. Our results indicated that at 18 h.p.i. with ECTV-MOS a significant decrease in the percentage of early and late apoptotic cells was observed in *beclin 1* siRNA-transfected L929 cells compared to wild-type L929 cells. The knockdown of *beclin 1* expression suppressed the apoptosis during ECTV-MOS replication cycle. It is not excluded that autophagy may have a role in regulation of apoptosis upon ECTV-MOS infection, because complex interrelationships between autophagy and the apoptotic cell death pathway are likely to exist. Regulators of apoptosis activation, such as sphingolipid, ceramide, the death-receptor signaling molecules, TRAIL and FADD, and the serine/threonine death kinases, DAPk and DRP-1 also function as regulators of autophagy activation (Levine and Yuan 2005). Moreover, autophagy genes may be involved in the death execution process, because knockdown of *atg7* or *beclin 1* suppressed death in L929 cells treated with the caspase inhibitor, ZVAD, and knockdown of *atg5* or *beclin 1* blocked death of $bax^{-/-}$ and $bak^{-/-}$ murine embryonic fibroblasts treated with etoposide or staurosporine (Shimizu et al. 2004; Yu et al. 2004). On the other hand, *beclin 1* silencing in L929 cells might allow Bcl-2 anti-apoptotic activity, independently of the physiological process of autophagy. Bcl-2 together with $BclX_L$ mainly reside in mitochondrial outer membrane and protect mitochondria against mitochondrial membrane permeabilization—a critical event responsible for caspase activation in the intrinsic apoptotic pathway (Kroemer et al. 2007).

In conclusion, we have shown that Beclin 1 is involved in the regulation of cell death during ECTV-MOS replication in permissive L929 cells. We speculate that there may exist viral proteins that regulate both autophagy and apoptosis through involvement of Beclin 1. Moreover, elevated autophagic activity may present an alternative pathway of cell death when apoptosis is inhibited.

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