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Increased formation of autophagosomes in ectromelia virus-infected primary culture of murine bone marrow-derived macrophages

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Summary. – Induction of autophagy by ectromelia virus (ECTV) in primary cultures of bone marrow-derived macrophages (BMDMs) was investigated. The results showed that ECTV infection of BMDMs resulted in increased formation of autophagosomes, increased level of LC3-II protein present in aggregates and extensive cytoplasmic vacuolization. These data indicate an increased autophagic activity in BMDMs during ECTV infection.

Keywords: ectromelia virus; macrophage; autophagy; Atg proteins

Introduction

Ectromelia virus (ECTV, the genus *Orthopoxvirus*, the family *Poxviridae*) is a causative agent of mousepox – a generalized infection in its natural host, the mouse. ECTV is used as a prototype to study the pathogenesis of poxviruses including variola virus (VARV), due to similarity in infectivity and high mortality rates following infection with either of the viruses in their respective natural hosts (Panchanathan *et al.*, 2006).

Autophagy is a catabolic process responsible for the degradation of aggregated proteins and removal of damaged organelles in animal cells. It may play an important role in viral replication and pathogenesis. During autophagy double-membrane vesicles, called autophagosomes, occupy a portion of cytoplasm or surround an organelle and then fuse with lysosomes where enzymatic degradation of their cargo occurs. Formation of autophagosomes is mediated by autophagy-related (Atg) proteins. Beclin 1 (Bcl-2-interacting protein 1), a mammalian homolog of yeast Atg6, is a key protein that regulates early steps of vesicle nucleation (Cao and Klionsky, 2007), whereas microtubule-associated protein 1 light chain 3 (LC3), a mammalian ortholog of yeast Atg8, regulates elongation of the autophagosomal membrane (Weidberg *et al.*, 2010).

Although autophagy can suppress the life cycle of certain viruses, this cellular machinery is also exploited by other viruses to enhance their replication and survival. Moreover, some viruses can induce autophagic cell death (type II programmed cell death, PCD-II) to facilitate the release of progeny virus particles at the end of the replication cycle. On the contrary, other viruses may influence autophagy to enhance survival of infected cells. Finally, some viruses modulate autophagy to enhance their virulence and pathogenesis and to inhibit anti-viral innate and adaptive immune response (Kudchodkar and Levine, 2009; Sir and Ou, 2010).

Since previously we have found induction of autophagy in L929 cells by ECTV (Martyniszyn *et al.*, 2011), we examined in this work whether ECTV is able to induce autophagy in other type of cells, in particular bone marrow-derived macrophages (BMDMs).

Materials and Methods

Virus. Highly infectious Moscow strain of ECTV (ECTV-MOS, ATCC 1374) was propagated and titrated by plaque assay in Vero

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Abbreviations: Atg proteins = autophagy-related proteins; Beclin 1 = Bcl-2-interacting protein 1; BMDMs = bone marrow-derived macrophages; ECTV = ectromelia virus; LC3 = microtubule-associated protein 1 light chain 3; p.i. = post infection

cell (ATCC CCL-81) monolayer. BMDMs were infected with ECTV at MOI 5. After 2, 4, 6, 12, and/or 18 hrs p.i. cells were harvested for further experiments. Uninfected BMDMs were used as controls.

Primary cultures of BMDMs. BMDMs were isolated from 6–8-week-old BALB/c mice, purchased from the Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology in Warsaw, Poland. Briefly, bone marrow cells were flushed out of tibias and femurs and resuspended in complete medium composed of Glutamax-I RPMI 1640 medium, 25 ng/ml macrophage-colony stimulating factor (M-CSF; Sigma-Aldrich), 10% FBS and 1% antimycotic antibiotic. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 5 days of culture, a homogeneous population of adherent macrophages was obtained.

Antibodies. The following antibodies were used in experiments: rabbit anti-LC3, rabbit anti-Beclin 1, mouse anti-β-actin, goat anti-rabbit-HRP, rabbit anti-mouse-HRP and goat anti-rabbit-FITC (all from Sigma-Aldrich).

Western blot analysis. BMDMs were lysed using the MCL1 kit (Sigma-Aldrich) according to the manufacturer's instructions. SDS-PAGE and Western blot analysis were performed as previously described (Martyniszyn *et al.*, 2011, 2013). Antibodies directed against LC3, Beclin 1 and β-actin were diluted 1:1000, 1:1000, and 1:5,000, respectively. Appropriate secondary antibodies were diluted 1:50,000. Quantification of the bands from the blots was performed using KODAK Image Station 4000MM Digital Imaging System. Protein levels were normalized to β-actin. The values were presented as mean ± SD (standard deviation) from 3 independent experiments. Statistical significance analysis of LC3-II and Beclin 1 levels was performed between control and ECTV-infected cells.

Immunofluorescent staining. BMDMs were cultured on chamber slides. After 18 hrs p.i. cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Intracellular staining of LC3 was performed as previously described (Martyniszyn *et al.*, 2011). Antibodies directed against LC3 and appropriate secondary antibodies were diluted 1:200 and 1:500, respectively. Nuclear DNA was counterstained with DAPI (Vector Laboratories). Images were recorded using confocal microscope (Olympus FV1000) or fluorescence microscope (Olympus BX60) equipped with Color View III cooled CCD camera and Cell^F software (Olympus).

Lysotracker Red staining. BMDMs grown on chamber slides were labeled with 1 μmol/l Lysotracker Red (Invitrogen) in PBS for 2 min at RT. Cells were visualized by Olympus BX60 fluorescence microscope using filters for rhodamine.

Statistical analysis. Quantitative data are expressed as mean ± SD from three independent experiments. Western blot experiments were analyzed using Mann-Whitney U test. The value of $P \leq 0.05$ was considered to be significant. All analyses were performed with Statistica 6.0 software (Statsoft Inc.).

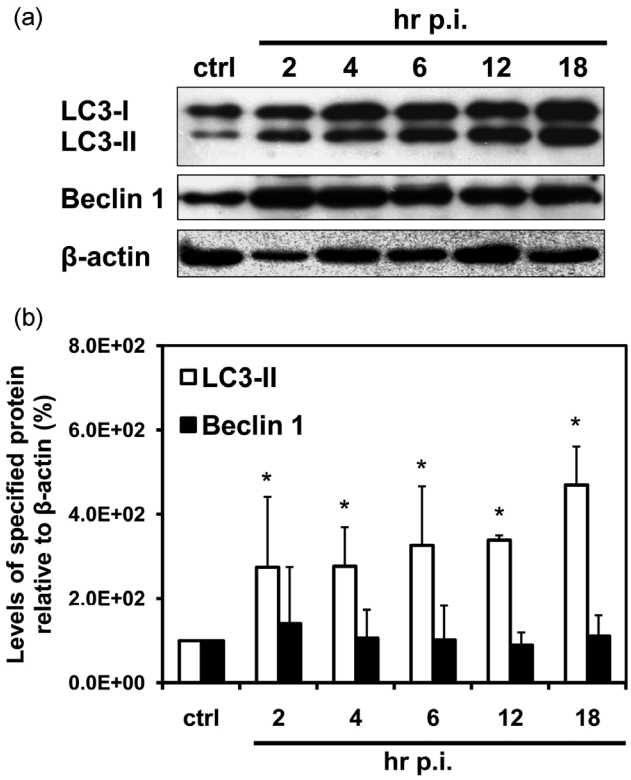

Fig. 1

Effect of ECTV infection on expression of Atg proteins in BMDMs

Western blot analysis. Western blot (a) and quantitative evaluation of Atg proteins (b). Statistically significant differences are marked with asterisk. Controls (ctrl).

Results and Discussion

Effect of ECTV infection on expression of Atg proteins in BMDMs

We used Western blot assay to determine the expression of LC3-II and Beclin 1 in control and ECTV-infected primary macrophages. Blotting of LC3 proteins revealed two bands: LC3-I (18 kDa) and LC3-II (16 kDa). Levels of LC3-II (which correlates well with the number of autophagosomes) and Beclin 1 (60 kDa) were measured relative to β-actin (42 kDa) loading control.

We found a statistically significant ($P = 0.0495$) increase in LC3-II/β-actin ratios during the replication of ECTV in BMDMs starting from 2 hrs p.i. compared to uninfected cells (Fig. 1a,b). The highest increase in LC3-II/β-actin ratio at least 4.5-fold above control was observed in BMDMs 18 hrs p.i. with ECTV. Meantime, Beclin 1/β-actin ratios were at the same level in both uninfected or infected BMDMs as revealed by densitometric analysis of protein bands (Fig. 1a,b).

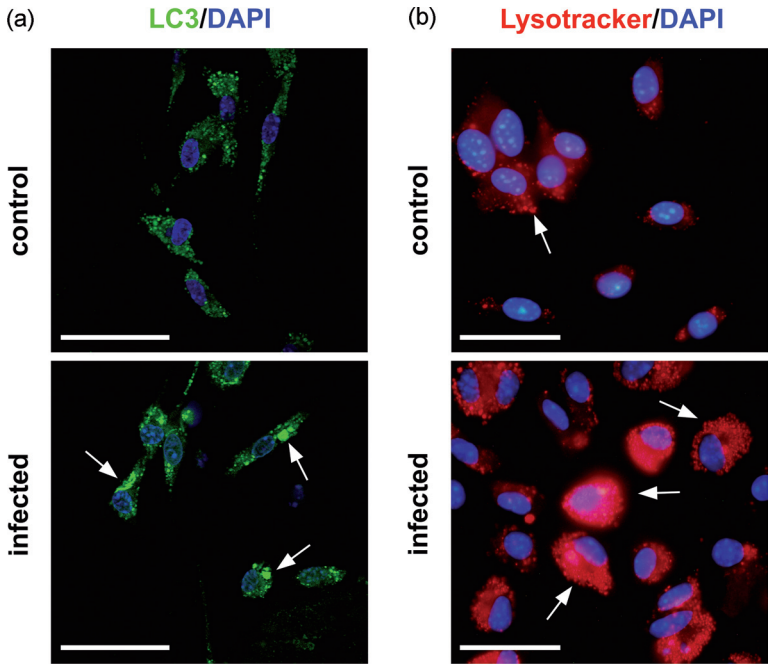

Fig. 2

Induction of autophagosome formation in BMDMs by ECTV

Fluorescent confocal microscopy 18 hrs p.i. Immunofluorescent staining of LC3 (green) (a), Lysotracker Red staining of vacuoles (arrows, red) (b) and DAPI counterstaing of DNA (blue). Bar: 50 μm.

Induction of formation of autophagosomes in BMDMs by ECTV infection

During later stages of infection, the occurrence of autophagosomes was additionally confirmed by fluorescence microscopy, which revealed that LC3 protein tended to aggregate in a punctate pattern within the cytoplasm of BMDMs infected with ECTV (Fig. 2a). Lysotracker Red staining demonstrated extensive cytoplasmic vacuolization in infected BMDMs (Fig. 2b). Taken together, these data suggest that during ECTV infection increased formation of autophagosomes may be related to increased autophagic activity in murine macrophages derived from bone marrow precursors.

Increased autophagic activity in BMDMs infected with ECTV may be linked to phagocytic activity and/or antigen presentation capabilities of macrophages. Autophagy is involved in MHC class II presentation of cytoplasmic and nuclear antigens, and potentially regulates MHC class I-mediated cross-presentation of exogenous antigens (Crotzer and Blum, 2009). In human macrophages, autophagy facilitated the presentation of herpes simplex virus 1 (HSV-1) endogenous antigens on MHC class I molecules and regulated the stimulation of $CD8^+$ T cells (English *et al.*, 2009). Recently, it has been found that the presentation of human cytomegalovirus (HCMV) endogenous protein, pUL138, through MHC class I pathway can also be mediated by autophagy (Tey and Khanna, 2012). Moreover, viral endogenous antigens, such as the Epstein-Barr virus nuclear antigen 1 (EBNA-1) can be delivered by autophagy from the cytosol or nucleus into the lysosomal vesicles. Lysosomal proteases then degrade these proteins and the resulting peptides are presented *via* the MHC class II pathway (Paludan *et al.*, 2005).

On the other hand, increased autophagy may facilitate replication and/or survival of ECTV in infected macrophages. Some viruses have evolved mechanisms to subvert autophagic machinery to facilitate replication in macrophages. It has been demonstrated that replication of human immunodeficiency virus 1 (HIV-1) was severely impaired in macrophages treated with the autophagy inhibitor 3-methyladenine (3-MA) and in Beclin 1 or Atg7 siRNA-transfected cells (Kyei *et al.*, 2009). Moreover, pharmacological induction of autophagy by rapamycin enhanced yields of virus released from macrophages (Kyei *et al.*, 2009). On the contrary, Campbell and Spector (2012) found that vitamin D could inhibit HIV-1 replication in macrophages through autophagy induction, and treatment of cells with bafilomycin A (BFA, an inhibitor of the lysosomal vacuolar-ATPase) and knockdown of Beclin 1 and Atg5 counteracted the inhibitory effect of vitamin D.

Because autophagy is involved in many biological functions, the exact role of autophagy in ECTV infection should be clarified in future studies. Moreover, studies are underway to determine ECTV induction of autophagy under *in vivo* conditions.

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