Antiviral Research 99 (2013) 238–244

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Increased mitochondrial functions in human glioblastoma cells persistently infected with measles virus

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ARTICLE INFO

Article history:
Received 15 April 2013
Revised 3 June 2013
Accepted 22 June 2013
Available online 2 July 2013

Keywords:
Measles virus
Persistent infection
Mitochondria
Mitochondrial membrane potential
Oxidative stress condition

ABSTRACT

Measles virus (MV) is known for its ability to cause an acute infection with a potential of development of persistent infection. However, knowledge of how viral genes and cellular factors interact to cause or maintain the persistent infection has remained unclear. We have previously reported the possible involvement of mitochondrial short chain enoyl-CoA hydratase (ECHS), which is localized at mitochondria, in the regulation of MV replication. In this study we found increased functions of mitochondria in MV-persistently infected cells compared with uninfected or acutely infected cells. Furthermore, impairment of mitochondrial functions by treatment with mitochondrial inhibitors such as ethidium bromide (EtBr) or carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) induced the cytopathic effects of extensive syncytial formation in persistently infected cells. These findings suggest that mitochondria are one of the subcellular organelles contributing to regulate persistent infection of MV. Recent studies showed mitochondria provide an integral platform for retinoic acid-inducible protein (RIG-I)-like cytosolic receptors (RLRs) signaling and participate in cellular innate antiviral immunity. Our findings not only reveal a role of mitochondria in RLR mediated antiviral signaling but also suggest that mitochondria contribute to the regulation of persistent viral infection.

1. Introduction

Mitochondria have been long recognized for their key role in cellular bioenergetics and apoptosis. However, mitochondria have now been shown to serve as a crucial platform for innate immune signaling (Arnoult et al., 2011; Koshiba, 2013; Wang et al., 2011; West et al., 2011). An important component of the host innate antiviral response is the interferon (IFN)-related responses. IFN production involves the recognition of pathogen-associated molecular patterns (PAMPs) including viral RNAs by Toll-like receptors (TLRs) and retinoic acid-inducible protein (RIG-I)-like cytosolic receptors (RLRs). RLRs comprise RIG-I and melanoma differentiation-associated gene 5 (MDA-5) and detect viral RNA in the cytosol. RIG-I and MDA5 contain two caspase recruitment domains (CARDs) that are required for interaction with the CARD domain of the mitochondrial antiviral signaling protein (MAVS; also known as IPS-1, Cardif and VISA). Some groups have reported that cytosolic viral signaling pathway through MAVS is specifically required for innate immune responses against viral infection (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). MAVS exists on the mitochondrial outer membrane and must be localized at the mitochondria to exert its function, implying that mitochondrial environment is required for signal transduction after activation of the RLRs. Moreover, several studies have shown that mitochondrial proteins controlling a mitochondrial dynamics played an important role in MAVS-mediated antiviral signaling (Koshiba et al., 2011a; Onoguchi et al., 2010).

On the other hand, we have been interested in mitochondria in a different view from MAVS-mediated antiviral signaling. We have previously established a human glioblastoma cell line persistently infected with mutant measles virus (MV), because MV provides one of the main paradigms of a long-term persistent infection by an RNA virus due to its involvement in the disease subacute sclerosing panencephalitis (SSPE) (Horta-Barbosa et al., 1969). Using persistently infected cells, we performed proteomic analysis to identify host cellular proteins that regulate a viral replication and to clarify the mechanisms of persistent infection. We found that the expression level of mitochondrial short chain enoyl-CoA hydratase (ECHS), which catalyzes the β-oxidation pathway of fatty acid, was specifically down-modulated in MV persistently infected cells. Moreover, ECHS knockdown by siRNA apparently impaired wild-

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http://dx.doi.org/10.1016/j.antiviral.2013.06.016

type MV replication (Takahashi et al., 2007). Therefore, the reduction of ECHS may keep low steady-state levels of virus replication in persistently infected cells. Since ECHS is localized in mitochondria matrix, we thought that mitochondria were one of the subcellular organelles contributing to the establishment of persistent infection.

Viral infection or some viral proteins cause altered mitochondria functions such as mitochondria membrane potential, ATP production, calcium uptake and reactive oxygen species production (El-Bacha et al., 2004; Piccoli et al., 2006; Silva da Costa et al., 2012). In this study, we examined the mitochondrial functions in acutely or persistently MV-infected cells. We found the remarkable increased functions of mitochondria in persistently infected cells compared with uninfected or acutely infected cells. Furthermore, impairment of mitochondrial functions by treatment with some mitochondrial inhibitors induced the cytopathic effects of extensive syncytial formation in persistently MV-infected cells. These observations suggest that mitochondrial physiological functions might contribute to the regulation of pathogenicity factors in MV. Here, we present a possible contribution of mitochondrial functions to the maintenance of persistent infection, and may provide clues about the molecular mechanism underlying the regulation of persistent infection.

2. Materials and methods

2.1. Cell cultures and viruses

Human glioblastoma cells A172 were grown in Eagle's minimum essential medium (MEM, Nikken BioMedical Laboratory, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin. Wild-type Edmonston strain meales virus (Rapp clone 5, R5-MV) was grown and titrated on Vero cells. A temperature sensitive mutant virus P-448 was established from Rapp clone 5 (Yamaji et al., 1975) and used to establish persistently infected cells (448-A172 cells) described previously (Takahashi et al., 2007). For virus titration, serial tenfold dilutions of cell supernatants and cell lysates were inoculated into each of four wells of Vero cells and then incubated for 7 days. After incubation, wells were scored for cytopathic effect (CPE) and we determined the dilution as $TCID_{50}$/ml at which 50% of the wells were infected.

2.2. Cell staining

For mitochondrial morphology assessment, cells were stained with MitoTracker-Red (Dojindo Molecular Technology, Kumamoto, Japan) at a final concentration of 50 nM for 30 min and then fixed with 4% formaldehyde for 15 min. For electron microscopic analysis, cells were fixed with 2.5% glutaraaldehyde and 1.6% paraformaldehyde. After fixed with 1% OsO_4, cells were dehydrated and embedded in Epon 812 (TAAB, Berks, England). Ultrathin sections were cut and stained with uranyl acetate and lead citrate for examination by H-700 electron microscope (Hitachi, Tokyo, Japan). For immunochemical analysis, cell cultures on culture slide were incubated with anti-MV-hemagglutinin monoclonal antibody (self preparation). After washing, they were overlaid with fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG antibody (Beckman Coulter, Inc., Brea, CA).

2.3. Measurement of cell growth

Cells were seeded at a density of 2×10^4 cells/well on 96-well microtiter plates. After 7 days of incubation, 50 μg of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma–Aldrich, St. Louis, MO, USA) was added to cell culture wells and the plates were incubated at 37 °C for 4 h. Acid isopropanol was added to all wells and mixed thoroughly to dissolve the dark blue crystals, and optical density was measured at 595 nM.

2.4. Measurement of mitochondrial functions

For measurement of mitochondrial membrane potential (MMP), cells cultured at low density on 24 well glass-bottom plate were incubated for 20 min at 37 °C with the following probes: 40 nM tetramethyl rhodamine methyl ester (TMRM, Molecular Probes, Invitrogen, Carlsbad, CA, USA), 1 μM Mito Tracker Green (MTG, Molecular Probes) and 2 μM Hoechst 34580 (Molecular Probes) to monitor MMP. Fluorescence was observed using a multidimensional imaging workstation (AS MDW, Leica Microsystems, Wetzlar, Germany) consisting of a tunable light source (Polychrome IV monochromator, Till Photonics, Gräfelfing, Germany), an inverted epifluorescence microscope (DM IRE2, Leica Microsystems) contained in a climate chamber maintained at 37 °C and a cooled charge-coupled device (CCD) camera (CoolSnap HQ, Roper Scientific, Princeton, NJ). A 0.35 Å∼ demagnifiying lens (Leica Microsystems) was inserted between the microscope and the CCD camera. The components were controlled by custom-made software written in C (Bloodshed Dev-C++) and LabVIEW (National Instruments, Austin, TX, USA).

The ATP level was quantitatively measured using an ATP assay kit with luciferin and luciferase according to the protocol provided by the manufacturer (TOYO B-Net Co., Tokyo, Japan).

The intracellular production of reactive oxygen species (ROS) was measured using the cell permeable probe 2′, 7′-dichlorofluorescein diacetate (DCFDA, Eugene, OR, USA). Briefly, cells infected with or without MV were incubated with 5 μM DCFDA for 30 min at 37 °C. Then cells were washed twice with PBS and resuspended in 0.5 ml of PBS and the fluorescence was immediately read on a Becton Dickinson FACScan with Cell Quest software (BD Biosciences, San Jose, USA).

Superoxide dismutase (SOD) activities were measured using a SOD assay kit-WST (Dojindo), according to the manufacturer's instructions. Briefly, cells were harvested and broken using Teflon homogenizer in PBS on ice. After the broken samples were centrifugated at 10,000*g* for 15 min at 4 °C, the supernatants were removed and pellets were resuspended to prepare sample solution.

2.5. Western blotting

Cells were lysed in 30 μl of lysis buffer (1% Noidet P-40, 140 mM NaCl, 2 mM EDTA, 20 mM Tris–HCl (pH 8.0), containing protease inhibitor cocktail (Nakalai tesque, Kyoto, Japan)) on ice for 15 min, and subsequently centrifuged for 15 min at 20,400*g*. Protein content in the lysates was measured using DC Protein Assay kit (Bio-rad, Hercules, CA, USA). The lysates were electrophoresed in SDS–PAGE under reducing conditions and transferred to a nylon membrane by means of semi-dry electrotransfer. The blots were probed with anti-MnSOD antibody (BD Biosciences, San Diego, CA, USA) or vaccinated human serum with MV or anti-β-actin antibody (Sigma) followed by peroxidase-conjugated goat anti-human or mouse IgG (Jackson ImmunoReseach, West Grove, PA, USA). Protein signals were detected using tetramethylbenzidine substrate kit (Vector, Burlingame, CA, USA).

2.6. Statistical analysis

Student's *t*-test was used to determine the significance of differences in means. A p value of <0.05 was considered significant.

3. Results

3.1. MV infection dose not induce alterations in mitochondrial morphology

To observe the effects of persistent infection with MV on mitochondrial morphology, 448-A172 cells and A172 cells infected with or without R5-MV were plated on culture slides. After 48 h of incubation, cells were stained with MitoTracker-Red and taken to detect mitochondrial morphogenetic alterations by fluorescence microscopy (Fig. 1 upper column). 448-A172 cells did not appear to affect mitochondrial morphological changes and mitochondria distribution compared to both A172 cells and R5-MV infected A172 cells. Transmission electron microscopy analysis also exhibited no differences of mitochondrial morphology among cells tested (Fig. 1 lower column).

3.2. Persistent infection with MV results in a increase in MMP and ATP content

The mitochondrial electron transport of respiratory chain generates MMP and drives the formation of ATP. MMP represents an important parameter for cellular metabolism and mitochondrial energy status. Therefore, we performed the experiments to assess the status of MMP during R5-MV infection at moi 1 for 24 h. MMP was measured using TMRM, a fluorescent lipophilic cation that accumulates in the mitochondrial matrix in a MMP dependent manner. TMRM uptake was normalized through determination of the fluorescence of MTG, which accumulated in mitochondria independently of MMP. TMRM loading was finally expressed as a ratio of TMRM fluorescence to MTG fluorescence. We observed that the red:green fluorescence intensity in R5-MV infected A172 cells were gradually decreased and at 20 h post infection red fluorescence almost disappeared especially in multinucleated syncytium, suggesting that the infection of A172 cells with R5-MV induced a loss of MMP (Fig. 2A and B). Furthermore, enhanced green fluorescent protein (EGFP)-expressing Edmonston strain MV (Ed-EGFP, (Schneider et al., 2002)) was used for confirmation of a loss of MMP in MV-infected cells (Fig. 2B). On the other hand, 448-A172 cells displayed higher red:green fluorescence intensity compared to non-infected A172 cells, which indicated that persistent infection with 448-MV caused a sustained elevation in MMP (Fig. 2C). We further determined ATP contents. Infection of A172 cells with R5-MV resulted in about 30% decrease in intracellular ATP level compared with non-infected A172 cells, whereas 448-A172 cells exhibited large amounts of ATP contents (Fig. 2D).

3.3. Persistent infection with MV results in an increased SOD activity and lowered production of ROS

ROS, including superoxide and its derivatives, are produced during various infections. Although production of ROS is an important host defense mechanism for killing invading pathogens, excessive ROS may be detrimental effect on host cells. The assessment of ROS levels in A172 cells infected with different moi of R5-MV was performed using the oxidant sensitive probe DCFH-DA. Flow cytometric analysis revealed that ROS production in R5-MV infected A172 cells was increased in moi-dependent manner at 24 h post infection. However, the level of ROS in 448-A172 cells was lower compared to R5-MV infected or uninfected A172 cells (Fig. 3A).

The first line of defenses against oxidative stress is SOD, which catalyze the dismutation of superoxide radical into H_2O_2. We observed that SOD activity was increased in 448-A172 cells compared to R5-MV infected or non-infected A172 cells. Western blot analysis also revealed that in comparison to R5-MV infected or non-infected A172 cells MnSOD protein expression was significantly increased in 448-A172 cells, which indicated that the increase in enzyme activity was proportional to the rise in protein level (Fig. 3B). These observations indicate the possibility that overexpression of MnSOD in 448-A172 cells might successfully scavenged ROS produced in mitochondria.

3.4. Impairment of mitochondrial functions cause cytopathic effects of extensive syncytial formation in 448-A172 cells

It was shown that mitochondrial functions such as MMP, ATP biosynthesis and SOD activity were elevated in 448-A172 cells. To examine whether these increased mitochondria functions were essential for maintaining a persistent infection, cells were treated with mitochondria inhibitor ethidium bromide (EtBr) or carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and assessed the potential of cell growth and syncytial formation. Low amounts of EtBr specifically accumulate in the mitochondria matrix, and affect mtDNA replication and lead to mtDNA depletion. This is followed by depletion of mtRNA and of mt-encoded proteins involved in oxidative phosphorylation, thus inducing mitochondrial dysfunction (King and Attardi, 1989). FCCP caused a complete

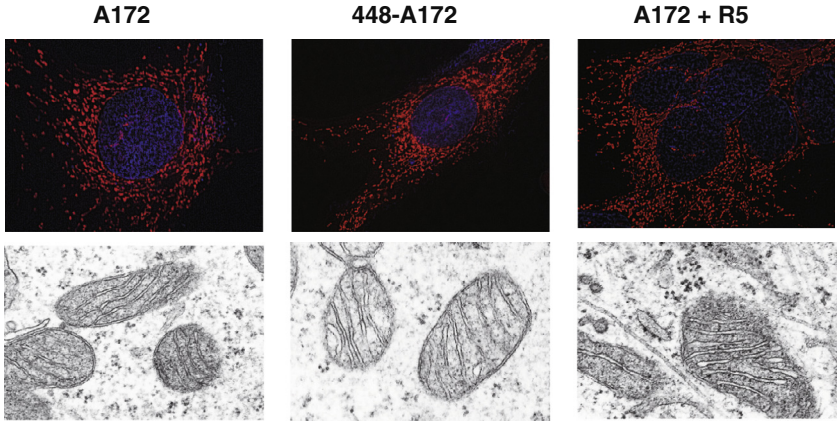

Fig. 1. Morphological assessment of mitochondria in persistently infected cells. A172 cells (left), 448-A172 cells (middle) and R5-MV-infected A172cells (right) were stained with MitoTracker-Red and fluorescence was observed with a fluorescence microscopy (upper column. ×400). Mitochondrial morphogenetic alterations were also confirmed by transmission electron microscopy analysis (lower column, ×24,000). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

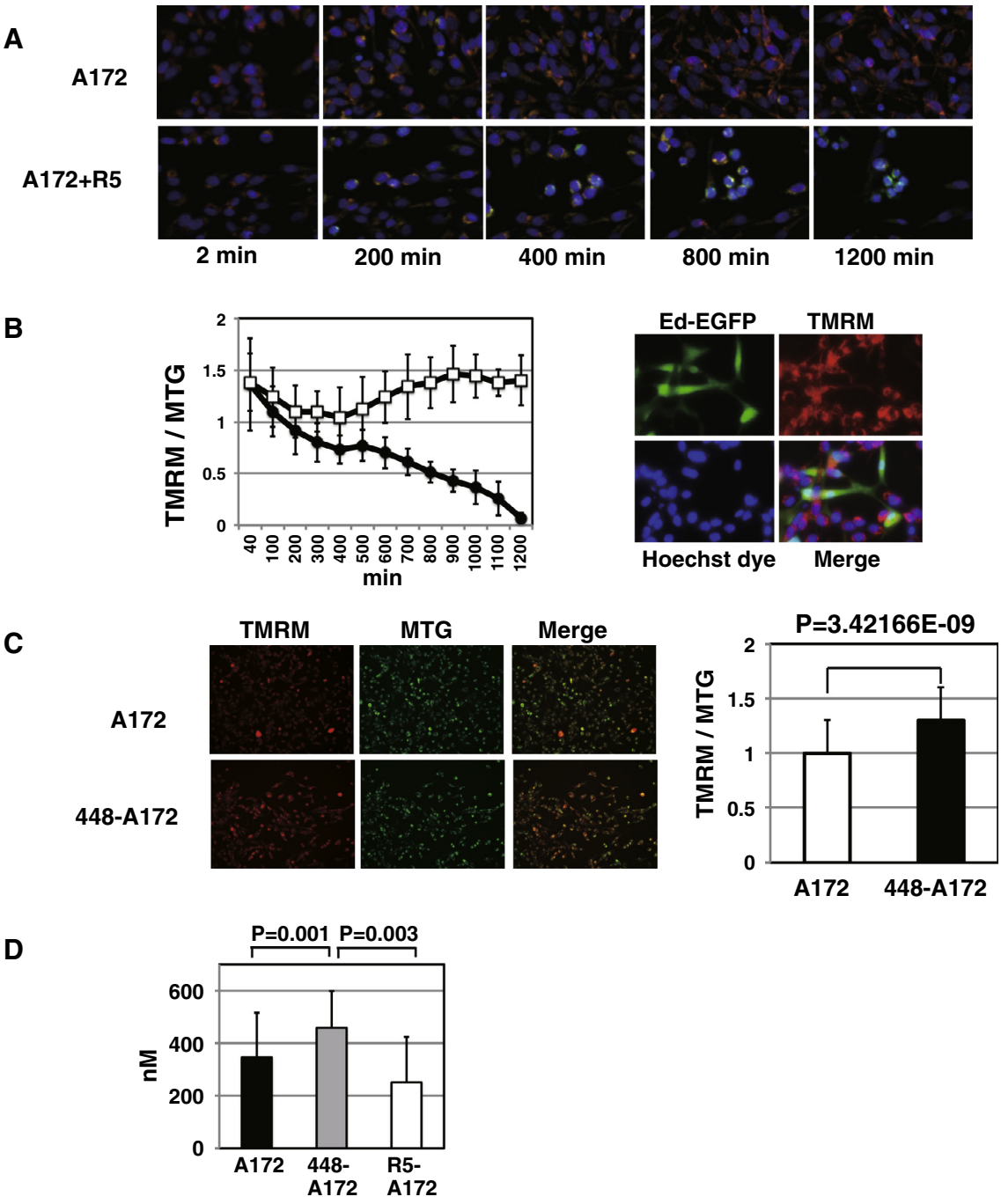

Fig. 2. Evaluation of MMP and ATP content in persistently infected cells. (A) A172 cells were infected with or without R5-MV at moi 1 for 24 h, followed by staining with TMRM (red), MTG (green) and Hoechst 34580 (blue), and fluorescence was observed with a multidimensional imaging workstation for 24 h. (B) TMRM loading observed in A was expressed as a ratio of TMRM fluorescence to MTG fluorescence (TMRM/MTG; left). Ed-EGFP was used for confirmation of a loss of MMP in MV-infected cells (right). Data are the means ± SD of values from four fields containing more than 100 cells. (C) Comparison of MMP between A172 cells (upper) and 448-A172 cells (lower). Data are the means ± SD of values from three fields containing more than 100 cells. (D) 448-A172 cells and A172 cells infected with or without R5-MV at moi 0.1 were cultured for 24 h and then subjected to the measurement of ATP content.

depolarization of the mitochondrial membrane, which resulted in induction of the maximal rate of respiration and inhibited production of ATP (Zubovych et al., 2010). As shown in Fig. 4A, 448-A172 cells were more susceptible to cytotoxicity of both EtBr and FCCP compared with A172 cells. Moreover, both EtBr and FCCP resulted in cytopathic effects of extensive syncytial formation in 448-A172 cells (Fig. 4B). Depletion of glucose from culture medium resulted in similar but a little detrimental effect on 448-A172 cells. Mitochondrial inhibitors and glucose depletion did not elevate the expression level of viral proteins (Fig. 4C), therefore, syncytial formation was not due to more abundant viral replication. These findings suggest that mitochondria might play a critical role in the regulation of cytopathic effect in MV.

4. Discussion

448-A172 cells produced a large amount of IFN-β in the culture supernatant more than twice relative to R5-MV infected A172 cells

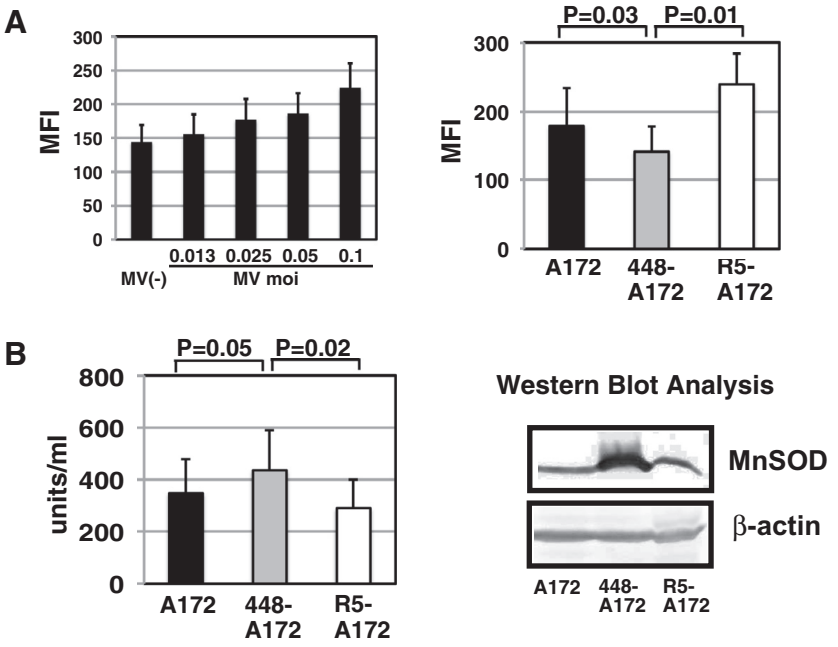

Fig. 3. Evaluation of SOD activity and ROS production in persistently infected cells. (A) A172 cells were infected with R5-MV with different moi and measured ROS production with oxidant sensitive probe DCFH-DA (left). Comparison of ROS production among A172 cells, 448A-A172 cells and R5-MV infected cells (right). Data are the means ± SD of values of at least three independent experiments. (B) Comparison of SOD activity (left) and protein expression of MnSOD (right) among A172 cells, 448A-A172 cells and R5-MV infected cells. Data are the means ± SD of values of at least three independent experiments.

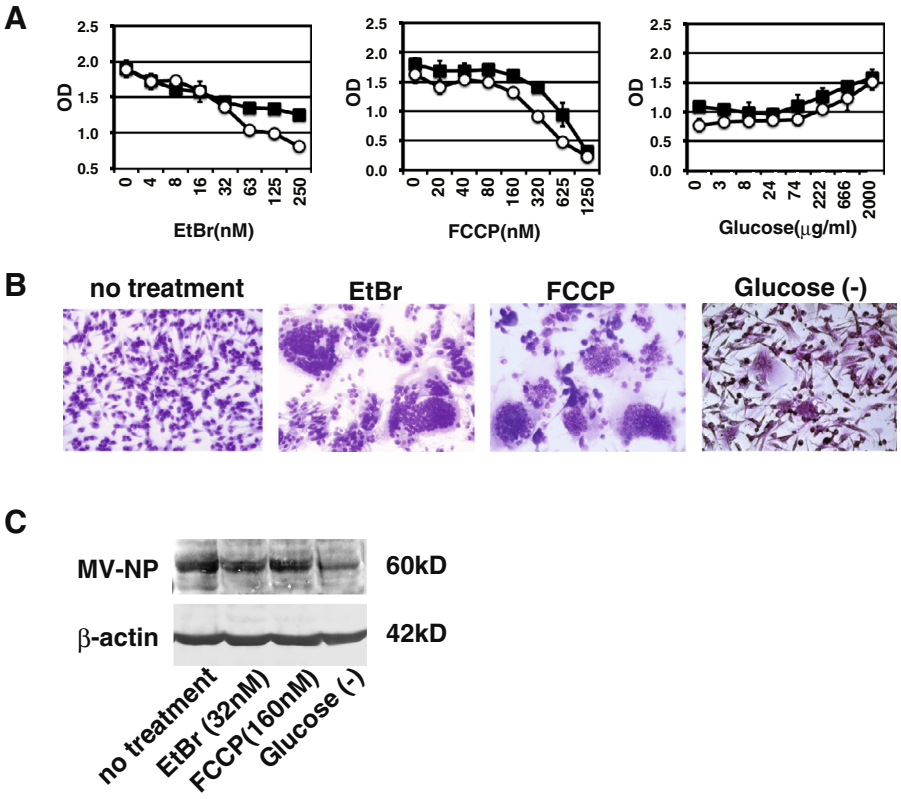

Fig. 4. Effect of EtBr, FCCP and depletion of glucose on the cell proliferation and syncytial formation in persistently infected cells. 448-A172 cells were cultured in the presence of 32 nM of EtBr or 160 nM FCCP, or in glucose-depleted culture medium. (A) After 7 days of incubation, cell proliferation of A172 cells (closed squares) and 448-A172 cells (open circles) were assessed by MTT method. Data are the means ± SD of values of at least three independent experiments. (B) After 10–14 days of incubation, cells were fixed with methanol and then stained with Giemsa solution. (C) Western blot analysis of MV nuclear protein (MV-NP) expression in 448-A172 cells cultured in the presence of EtBr or FCCP or in glucose-depleted culture medium for 14 days.

(data not shown). High titer of IFN-β might contribute to lower level of viral replication in persistent infected cells. However, we have previously shown that culture supernatant from 448-A172 cells containing IFN-β and measurable amounts of viruses (10_4 $TCID_{50}$/ml) induced syncytial formation for intact A172 cells (Takahashi et al., 2007). These findings suggest that such persistent infection dose not depend only on the massive production of IFN-β but rather on another cellular factors. A hallmark of persistent infection is an excellent intracellular status for both stable and low-level virus replication and development of resistance to virus pathogenicity. Previously, we have shown that ECHS might contribute to the regulation of virus replication. In this study we observed that persistently infected cells exhibited significantly enhanced mitochondrial functions, whereas acute infection induce mitochondrial dysfunctions. Moreover, impairment of mitochondrial functions caused cytopathic effects of extensive syncytial formation in persistently infected cells. These observations suggest that mitochondria functions might play an important role in the regulation of virus pathogenicity.

Many viral infection results in an increased ROS production and this oxidative stress are also implicated in the pathogenesis and morbidity of many virus infections (Lang et al., 2013; Vlahos et al., 2012). To protect the cells from oxidative stress, cells have an enzymatic and non-enzymatic scavenging system (Chen et al., 2011; Gac et al., 2010). In this study, we observed a significant increase in the expression levels of MnSOD in 448-A172 cells, correlating with a decrease of intracellular ROS. MnSOD, which resides in the mitochondria, is the most important antioxidant defense system, because mitochondria are the main source for free-radical production. Yoshinaka et al. have also reported that induction of MnSOD upon sindbis virus infection could be involved in the establishment of the persistent infection in infected cells (Yoshinaka et al., 1999). Thus lowered oxidative stress conditions might represent one of the effective factors contributing to prolonged cell survival.

The most prominent roles of mitochondria are to produce cellular energy, and MMP is essential for ATP synthesis by oxidative phosphorylation. Most viral infections promoted a reduction in MMP and cellular ATP content, because energy demand may be required to the synthesis of viral proteins and this alteration in energy balance may be associated with the pathogenesis of viral infection (El-Bacha et al., 2004, 2007). Moreover, recent study suggested that dissipation of MMP resulted in defective MAVS-mediated antiviral signaling (Koshiba et al., 2011b; Sasaki et al., 2013). Here we found that acute infection with R5-MV induced a decrease in MMP and ATP content, whereas 448-A172 cells exhibited a sustained elevation in MMP and large amounts of ATP content. However, treatment of 448-A172 cells with mitochondrial inhibitors caused cytopathic effects of extensive syncytial formation. These findings suggest that elevated MMP and ATP content might be essential for the development of resistance to viral infection.

We observed that persistently infected cells had high-quality mitochondria and impairment of mitochondrial functions caused the cytopathic effects of extensive syncytial formation. These findings suggest that mitochondria functions might be directly or indirectly involved in the regulation of host factors associated with MV mediated cell fusion. Although little is known concerning host factors associated with MV-mediated cell fusion, it has been reported that lipid rafts provide a cellular location for MV assembly in infected cells (Robinzon et al., 2009). Moreover, host factors that allow MV to establish persistent infection have been reported to include altered regulation of lipid metabolism (Anderton et al., 1981; Takahashi et al., 2007). Lipid metabolism is largely dependent on mitochondria. Therefore, our observations suggest that comprehensive mitochondrial functions, including energy metabolism, lipid metabolism and antioxidant activity, might be contributed to the establishment and maintenance of persistent virus infection.

Collectively, we described here for the first time of functional alterations in mitochondria, related to energy condition and antioxidant status of persistent infected cells. Although we have no direct evidence for how mitochondrial functions contributes to the regulation of syncytial formation, these results might contribute to the understanding of the maintenance of persistent infection and possibly to develop novel anti-viral drugs concerned with mitochondria functions.

Acknowledgments

We thank Dr Kaoru Takeuchi for useful technical support and discussion. This study was supported in part by grants from the Japanese Health Sciences Foundation, from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

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