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Everolimus delayed and suppressed cytomegalovirus DNA synthesis, spread of the infection, and alleviated cytomegalovirus infection

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ABSTRACT

Everolimus is an inhibitor of mammalian target of rapamycin (mTOR) and reduces the risk of cytomegalovirus (CMV) infection in transplant recipients. Everolimus inhibits mTOR complex 1, which regulates factors involved in several crucial cellular functions and is required for CMV replication. However, it is not clear how everolimus regulates CMV replication and prevents and alleviates CMV infection. Effects of everolimus on CMV infection, spread, and DNA synthesis and release from infected cells were assessed by plaque formation, infectious centre assay, real-time PCR of infected cells, and culture supernatant in CMV-infected cultures with and without everolimus. Everolimus enhanced plaque formation by 3.6 times, but the size of the plaques was reduced to 36.4% of untreated cultures in the absence of a pretreatment period. Everolimus reduced viral adsorption but enhanced the replication efficiency of inoculated virus, resulting in an increase in plaque number in the early phase of infection. Preinfection treatment of cells with everolimus efficiently exhibited its antiviral efficacy, and everolimus delayed and suppressed viral DNA synthesis and release from infected cells. Everolimus had suppressed the spread of infection and reduced the number of total infected cells to 40% of untreated cells on day 9, indicating reduction of the size of CMV lesions to one-sixth in 2–3 replication cycles. Preinfection treatment of the cells with everolimus augmented its suppressive effect on CMV infection and replication. Everolimus reduced the total number of infected cells and limited the CMV lesions, and this reduction in the spread of CMV infection would alleviate CMV infection in transplant recipients.

1. Introduction

Transplant recipients are maintained on immunosuppressive therapy to prevent rejection and loss of the allograft. The major immunosuppressive agents that are available in various combination regimens are glucocorticoids, azathioprine, mycophenolate mofetil (MMF), mizoribine, cyclosporine, tacrolimus, everolimus, rapamycin, and belatacept (Halloran, 2004; Ventura-Aguiar et al., 2016).

Everolimus is an inhibitor of mammalian target of rapamycin (mTOR) that inhibits mTOR complex 1 (mTORC1) and regulates factors involved in several crucial cellular functions, such as protein synthesis, regulation of angiogenesis, lipid biosynthesis, mitochondrial biogenesis and function, cell cycle, and autophagy (Granata et al., 2016). Many clinical studies have shown that everolimus or rapamycin decreases the risk of CMV infection in transplant recipients (Andreassen et al., 2014; Brennan et al., 2011; Hocker et al., 2016; Lehmkuhl et al., 2009; Nashan et al., 2012; Radtke et al., 2016; Strueber et al., 2016; Tedesco-Silva et al., 2015; Vigano et al., 2010; Vitko et al., 2005; Webster et al., 2006). mTORC1 regulates translation by phosphorylation of eIF4E-binding protein and p70 S6 kinase, and cell growth via phosphorylation of p70 S6 kinase and eukaryotic initiation factor binding protein. CMV replication is dependent on the activation of mTOR and is inhibited by rapamycin and everolimus (Clippinger et al., 2011a, b; Moorman and Shenk, 2010; Roy and Arav-Boger, 2014). Although extensive studies on the effects of everolimus on CMV without pretreatment have been performed, the underlying mechanisms for decreased episodes of CMV replication are not well understood (Clippinger et al., 2011a, b; Kudchodkar et al., 2007; Kudchodkar et al., 2004; Moorman and Shenk,

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2010; Roy and Arav-Boger, 2014). The anti-CMV activity of mTOR inhibitors has been shown immunologically by their mechanism of action, an improvement in CMV-specific CD8/CD4 T cell responses (Havenith et al., 2013; Roy and Arav-Boger, 2014).

We have characterised the effects of cyclosporine, tacrolimus, prednisolone, MMF, mizoribine, and azathioprine on CMV plaque formation and replication in treated cells (Kuramoto et al., 2010; Shiraki et al., 1990). Prednisolone and cyclosporine enhance CMV growth, and tacrolimus slightly suppresses CMV growth (Shiraki et al., 1991a). These effects on CMV growth by immunosuppressants may be due to a modification of cellular metabolism influencing CMV growth. Mizoribine (Halloran, 2004), which is used mainly in Japan (Ushigome et al., 2016; Yoshimura et al., 2013) and China (Shi et al., 2017), and MMF are inosine monophosphate dehydrogenase inhibitors and used for transplantation. Mizoribine dose-dependently suppresses CMV growth, whereas MMF does not. Mizoribine treatment of CMV-infected cultures resulted in the isolation of two mizoribine-resistant mutants, indicating the direct inhibition of CMV function for replication by mizoribine (Kuramoto et al., 2010).

In this study we characterised the effects of everolimus on CMV infection in vitro and found the importance of pretreating cells with everolimus to show its pharmacological action on CMV replication by reduced adsorption and reduced and delayed viral DNA synthesis, resulting in formation of smaller plaques. Thus, the suppressive action of everolimus as an anti-CMV drug that reduces the size of CMV lesions may reduce the risk of CMV infection in transplant recipients maintained on everolimus.

2. Materials and methods

2.1. Cells and viruses

Human embryonic lung (HEL) cells were grown and maintained in Eagle's minimum essential medium supplemented with 10% and 2% foetal bovine serum, respectively. A cell-free Towne strain of CMV was obtained by rapid freezing and thawing of infected cultures three times, followed by centrifugation, and storage at −70 °C until use in the experiments; it was propagated in HEL cells (Aiba et al., 2017; Kuramoto et al., 2010; Yajima et al., 2015). An intravenous immunoglobulin preparation, Venoglobulin IH, as an anti-CMV antibody, was purchased from Japan Blood Product Organization Co., Tokyo, Japan, and contained 50 mg/mL with a neutralization antibody titre of 1:163 (Aiba et al., 2017; Yajima et al., 2015).

2.2. Virus infection and treatment with everolimus

The effects of everolimus (dissolved in ethanol; Selleck Co., JP, Osaka, Japan) on CMV growth and spread of infection were assessed by plaque assays (Kuramoto et al., 2010; Shiraki et al., 1990, 1991a, 1991b). Confluent HEL cells in 60-mm dishes were infected with 100 plaque-forming units (PFU) of CMV in 0.2 mL for 1 h, and then 1% methylcellulose nutrient medium containing 0–1000 ng/mL everolimus was overlaid on the infected cells. The cells were incubated at 37 °C for 8 d and fixed with 5% neutral formalin, followed by staining with 0.03% methylene blue. The number of PFUs was counted under a dissecting microscope.

Because plaque size depends the number of infected cells, the reduction in plaque size was quantitated by the reduction in the number of infected cells in everolimus-treated cultures. Spread of CMV infection to neighbouring cells was monitored by the number of infected cells in the infected cultures assessed by the infectious centre assay (Aiba et al., 2017; Shiraki et al., 1990, 2011; Yajima et al., 2015). Cells were infected with CMV at 100 PFU per well in 6-well plates and overlaid with 1% methylcellulose nutrient medium. The infected cultures were incubated in semisolid methylcellulose medium to confine the cell-to-cell spread and prevent infection of remote cells via released virus. At the indicated times, the infected cultures were washed with maintenance medium to remove methylcellulose, and the cells were treated with trypsin and suspended in 1 mL of medium. Then, 0.1 mL of serially diluted suspensions was inoculated on fresh HEL cells in 6-well dishes in duplicate and overlaid with 1% methylcellulose medium. After 8 d of incubation the number of plaques was counted, and the spread of infection was determined by the increase in the number of infected cells. The sizes of plaques were determined using the ImageJ software (https://imagej.nih.gov/ij/download.html) of the US National Institutes of Health, and the cultures with more than 34 plaques were used for comparison of the effect of each treatment on plaque formation. .

Intracellular virus production was assessed by one-step growth in cells treated with drugs, as reported previously (Kuramoto et al., 2010; Shiraki et al., 1990). HEL cells in 25 cm^2 plastic flasks were infected with 2 PFU/cell of CMV for 1 h. The cells were washed three times with maintenance medium and incubated in this medium containing everolimus from 0 to 100 ng/mL for 3 d. Then, the cells were washed three times, moved to 5 mL of fresh maintenance medium, frozen and thawed three times, and centrifuged at 3000 revolutions per minute (rpm) for 10 min. Serially diluted supernatants were inoculated onto HEL monolayers in 60-mm plastic dishes and overlaid with nutrient methylcellulose medium. After incubation for 8 d, the number of plaques was counted.

2.3. Isotopic labelling and immunoprecipitation of cells treated with everolimus

CMV-infected cells were subjected to immunoprecipitation to identify the effect of everolimus on viral protein synthesis using anti-CMV human serum. CMV-infected cells were treated with various concentrations of everolimus and 200 μg/mL of phosphonoformic acid (PFA), then labelled with 50 Ci/mL of [^{35}S]-methionine and [^{35}S]-cysteine (37 T Bq/mmol, GE Healthcare Bio-Science Corp., Piscataway, NJ) in medium without methionine 48–64 h after infection. The labelled cells were sonically lysed in radioimmunoprecipitation assay buffer (20 mM-Tris-HCl pH 8-0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaC1 and 1 mM phenylmethanesulfonyl fluoride), followed by centrifugation at 35,000 rpm for 1 h. The supernatants were mixed with anti-CMV human serum, and the immune complexes were separated on Protein G-Sepharose CL-4B and solubilised in the sample buffer (50 mM-Tris-HCI pH 8.2, 1% SDS, 10% glycerol and 0.01% phenol red) with 5% 2-mercaptoethanol by boiling for 3 min (Shiraki and Hyman, 1987; Shiraki et al., 1991c). The immune complexes were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography.

2.4. Virus adsorption in everolimus-pretreated cells

Because an everolimus concentration of 5 ng/mL showed enhanced effects on plaque formation with reduced plaque size and was comparable to the plasma concentration in transplant patients (Carvalho et al., 2011; Kovarik et al., 2004), this concentration was used for further experiments. Adsorption of CMV was tested in cells pretreated with and without 5 ng/mL everolimus for 24 h; cells were infected in 24-well plates at 2 PFU/cell and further treated with 5 ng/mL everolimus for 24 h. Then viral DNA was extracted from the cells using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) and subjected to quantitative PCR. The amount of viral DNA in everolimus-treated cells was compared with that in untreated cells.

2.5. Effect of preinfection with everolimus and treatment length on plaque formation

Adsorption and spread of CMV infection was compared in cells with and without treatment of 5 ng/mL everolimus. Effects of preinfection

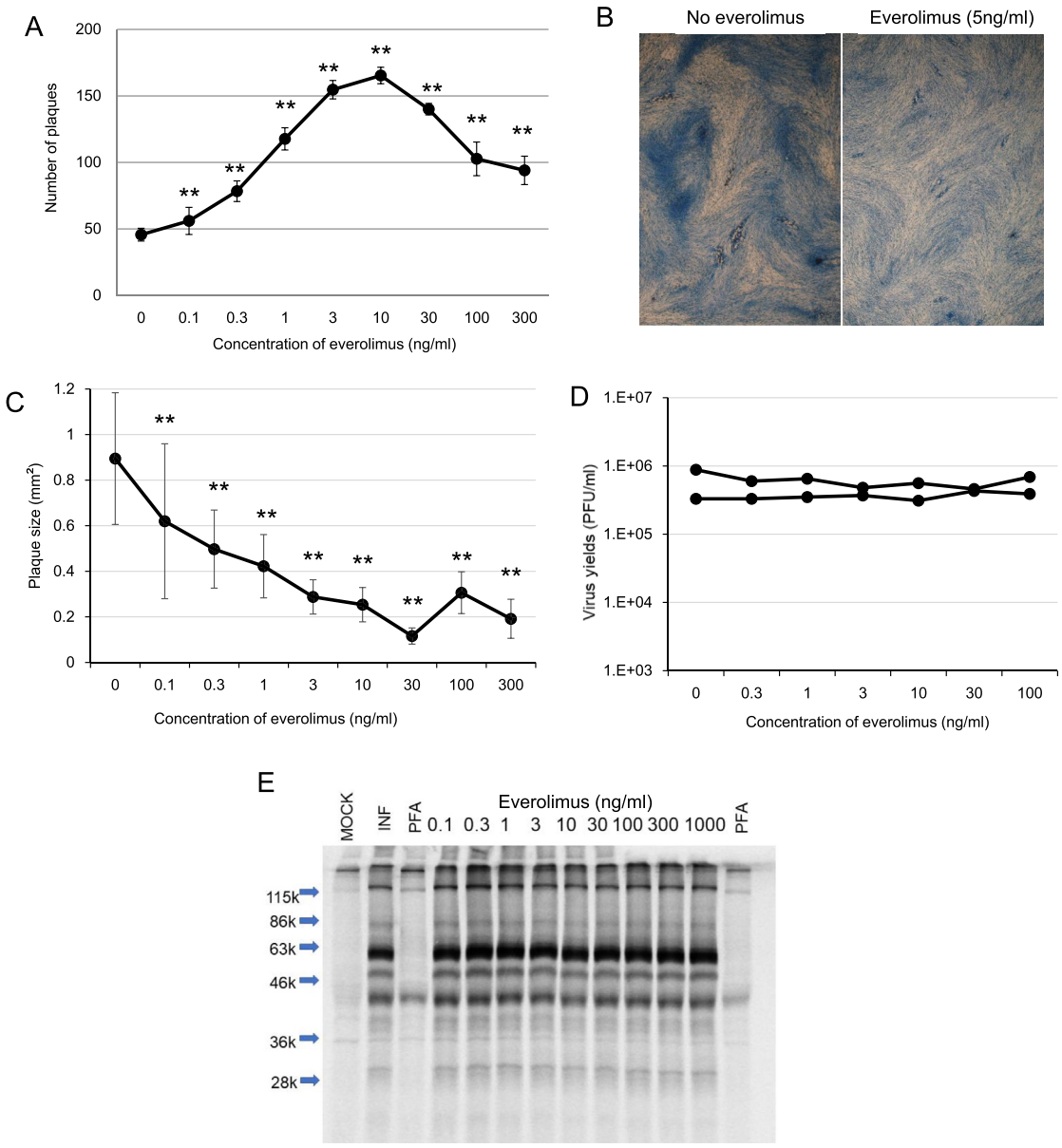

Fig. 1. Effect of everolimus without pretreatment of the cells. A. Everolimus increased plaque formation in a bell-shaped curve with the peak at 3–10 ng/mL (n = 3). **indicates statistical significance of untreated cells in plaque number against those treated with everolimus by one-way ANOVA, followed by Dunnett's multiple comparison ($P < 0.01$). B. The plaque sizes of cells with no everolimus- and those treated with 5 ng/mL everolimus 8 days after infection. C. Effect of everolimus without pretreatment of the cells. The plaque sizes of each treated culture were measured using ImageJ software, and everolimus increased plaque formation (Fig. 1A). ** indicate a statistically significant reduction in the plaque size of untreated cells due to the treatment with everolimus by one-way ANOVA, followed by Dunnett's multiple comparison ($P < 0.01$). D. Viral yields of the infected cells in one-step growth at 72 h after infection and treatment with various concentrations of everolimus as assessed by plaque titration in two experiments (n = 4). E. Viral protein synthesis in infected cells treated with various concentrations of everolimus and labelled with ^{35}S-methionine and cysteine at 48–64 h after infection. Viral proteins were visualised after immunoprecipitation with anti-CMV antibody and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography. The abbreviations Mock and INF indicate uninfected and CMV-infected, respectively; phosphonoformic acid (PFA) inhibits viral DNA synthesis, and proteins detected were early proteins.

and postinfection treatments with 5 ng/mL everolimus for 24 h on plaque formation were analysed by the plaque numbers. Confluent HEL cells in 60-mm dishes were treated with 5 ng/mL everolimus for 24 h before and after infection with 100 PFU of CMV for 1 h, and cultures with no, preinfection, postinfection, and combined treatments with everolimus were overlaid with 1% methylcellulose nutrient medium without everolimus and incubated further for 8 d. The number of plaques was counted, and the effect of their combined treatments on plaque formation was analysed.

To examine the effect of the length of pretreatment with everolimus on plaque formation, six sets of 6-well dishes were treated with 0 and 5 ng/mL everolimus for 1–5 d before infection, and all these dishes were infected at the same time. The infected cultures were incubated for 8 d without everolimus, and the effect of the length of pretreatment was evaluated by the number of plaques.

2.6. Viral DNA synthesis and virus release in CMV-infected cell cultures treated with everolimus

Because viral adsorption was suppressed by pretreatment of cells with everolimus, the cell surface and intracellular transport of virus might have been modified. Therefore, viral DNA synthesis and

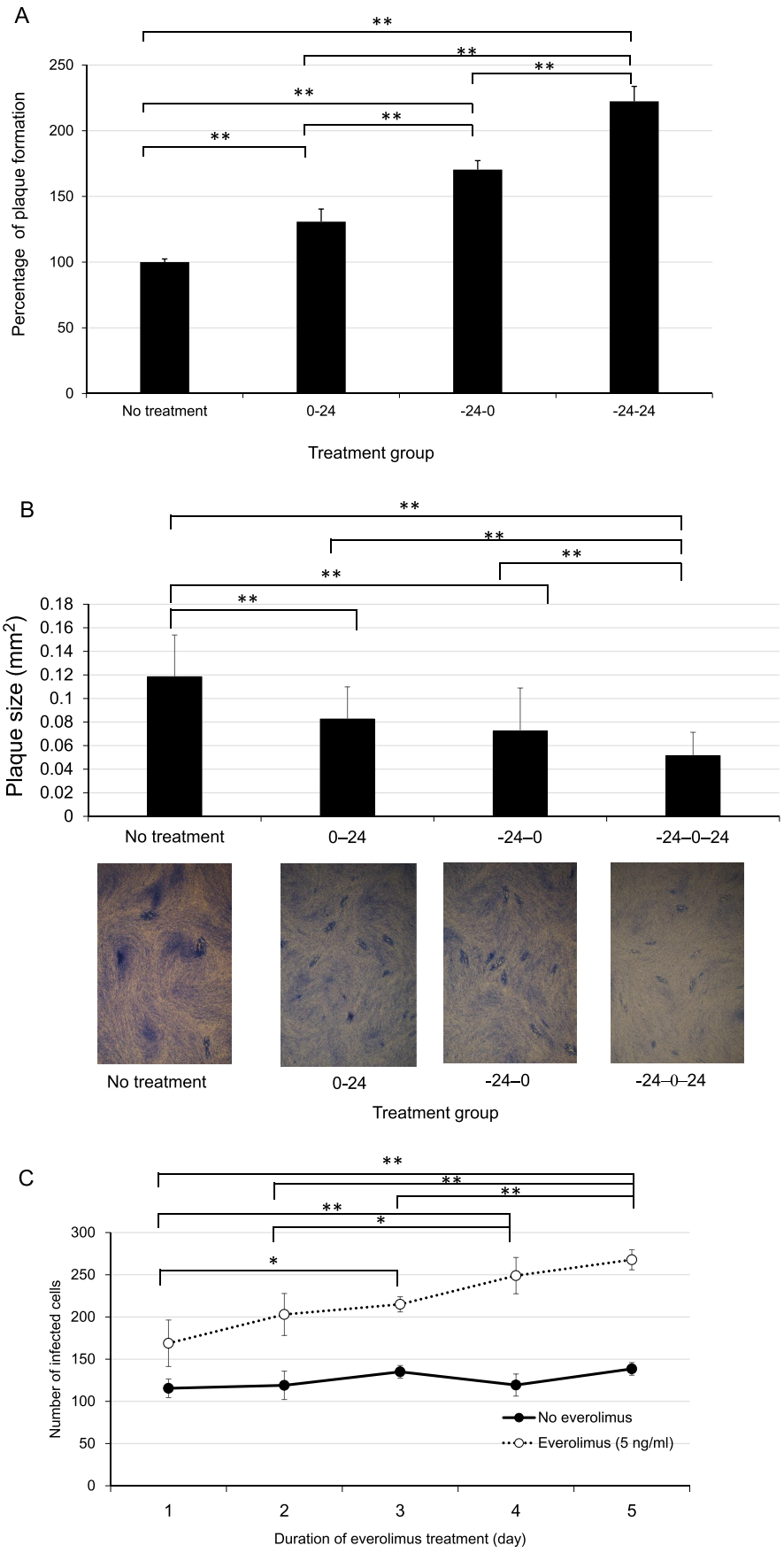

Fig. 2. Effect of preinfection treatment with everolimus for 24 h on CMV replication. A. Effect of preinfection and postinfection treatment with 5 ng/mL everolimus for 24 h on plaque formation. Panel A shows no everolimus treatment, and 0–24, -24–0, and -24–0–24 indicate everolimus treatment for 24 h after infection, 24 h before infection to time of infection, and 24 h before infection and 24 h after infection. respectively. The mean plaque numbers (n = 6) are shown as the mean ± standard deviation (SD) by error bars, and significant differences among treatment lengths (days) were analysed by one-way ANOVA followed by the Bonferroni/Dunnett multiple comparison method. **, $P < 0.01$. The sizes of plaques are shown under the plaque number in the treatment groups. Panel B shows the sizes of plaques measured using ImageJ software without everolimus treatment, and Panel A shows plaques with treatment for 0–24, -24–0, or -24–0–24 h after infection. The number of plaques receiving each treatment is shown under Panel B. The size of plaques is smaller in 24-h treated cells (0–24 and -24–0) and smallest in 48-h-treated cells (-24–0–24). C. Effects of the length of everolimus treatment on the number of plaques. Cells in 6-well plates were treated with or without 5 ng/mL of everolimus for 5, 4, 3, 2, or 1 day before virus infection, then the cells were washed three times to remove everolimus, and all sets of cells were infected with the same virus solution at the same time after washing. The cells were incubated in methylcellulose nutrient medium for 8 d. The number of plaques was counted after fixation and staining. The number of infected cells in each treatment was expressed as the mean ± SD (n = 6) in the control cultures without everolimus, and significant differences among treatment lengths (days) were analysed by one-way ANOVA followed by the Bonferroni/Dunnett multiple comparison method. *, $P < 0.05$; **, $P < 0.01$.

extracellular released viral DNA were assayed to assess the effect of everolimus on viral DNA synthesis, and the intracellular transport and release of viral DNA as viral particles in cells treated with and without 5 ng/mL everolimus were determined. One set of cell cultures in 24-well plates were treated with 5 ng/mL everolimus for 24 h before infection, infected with 2 PFU/cell of CMV, and further cultured with 5 ng/mL everolimus. Another set of cell cultures were infected with CMV at 2 PFU/cell and cultured without pre- and posttreatment with everolimus. The infected cells and the culture supernatants in the infected cultures treated with and without everolimus were harvested every 4 h after infection and the amount of viral DNA in the cells and the viral DNA released into the culture supernatants was determined by quantitative PCR.

2.7. Real-time PCR for quantitation of viral DNA

To measure CMV genome copy number, 2 μL of 50 μL extracted DNA solution in a reaction mixture was used for real-time PCR with a human cytomegalovirus (HCMV) UL54 DNA polymerase gene-specific primer set, forward primer (5′-GCGCGTACCGTTGAAAGAAAAGCATAA-3′) and reverse primer (5′-TGGGCACTCGGGTCTTCATCTCTTTAC-3′) (SYBR Premix Ex Taq; Takara Bio, Otsu, Shiga, Japan) (Daikoku et al., 2013). Before the quantitative PCR, the mixture was incubated at 95 °C for 10 s. The quantitative PCR reaction was performed at 95 °C for 5 s and 60 °C for 30 s for 45 cycles, and the amounts of PCR products were monitored with a Takara Dice thermal cycler for the real-time PCR system and analysed with Takara Real-time PCR software (Takara Bio, Otsu, Shiga, Japan).

2.8. Statistical analysis

Comparison of the plaque number and absorbed DNA copy number between groups cultured with and without everolimus was analysed by Student's *t* test. The plaque numbers and plaque sizes of each treatment with and without everolimus were analysed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison method between untreated and everolimus-treated cultures and the Bonferroni/Dunnett multiple comparison method for each treatment. Comparison of the numbers of the DNA copies in the cells and culture supernatants with and without everolimus during the time course was analysed by the repeated-measures ANOVA. The differences were considered statistically significant if values of $P < 0.05$.

3. Results

3.1. Effect of everolimus on plaque formation, virus yields, and viral protein synthesis without pretreatment

Fig. 1A shows the profile of the concentration-dependent effect of everolimus on plaque formation: the number of plaques increased in a bell shape in relation to treatment with everolimus, with a peak of 3.6 times without everolimus. However, the plaque sizes, as measured by ImageJ software, of everolimus-treated cultures were smaller than those of untreated cultures (Fig. 1B). Everolimus dose-dependently reduced the plaque size to 36.4% of that of untreated cultures, as shown in Fig. 1C. Everolimus had little effect on viral yields in the infected cells, as shown in Fig. 1D, in contrast to the enhanced plaque formation (Fig. 1A). The little effect of everolimus on virus yields was confirmed by quantitating the amount of intracellular viral DNA by real-time PCR (data not shown). Plaque formation was modified, but virus yields were not affected by everolimus at any concentration. The effects of everolimus on viral protein synthesis were examined, but there was no obvious alteration in the pattern and amount of viral protein synthesis detected by immunoprecipitation, as shown in Fig. 1E. Although these experiments were performed without pretreatment with everolimus, everolimus increased the number of plaques but reduced the size of plaques without affecting virus yields and protein synthesis.

3.2. Effect of preinfection treatment with everolimus on viral adsorption and plaque formation

The everolimus concentration used for the remaining experiments was 5 ng/mL because this concentration enhanced plaque formation with small plaques, as shown in Fig. 1, and was comparable to the plasma concentration in transplant patients (Carvalho et al., 2011; Kovarik et al., 2004).

Fig. 2A shows the effects of preinfection treatment and postinfection treatment, as well as their combination, of everolimus for 24 h on plaque formation. Preinfection treatment was more efficient in enhancing the plaque formation than postinfection treatment. The order of the enhancing effect of everolimus on plaque formation was preinfection and postinfection treatment (222.4 ± 11.3%), preinfection treatment (170.4 ± 6.9%), postinfection treatment (130.8 ± 9.6%), and no treatment (100%), as shown in Fig. 2A. The plaque size of everolimus-treated cells was compared with that of untreated cells, and the plaque size was smaller in everolimus-treated cells than in untreated cells (Fig. 2B). Enhancement of plaque formation was 3.46 ± 1.71 times higher in preinfection and postinfection treatment than in no treatment in three independent experiments. Everolimus treatment produced an increase in the number of plaques and a reduction in plaque size, depending on the concentration and treatment duration. Preinfection treatment with 5 ng/mL everolimus for 24 h was important for analysing the effect of everolimus on CMV-infected cells, in addition to postinfection treatment.

3.3. Importance of length of pretreatment with everolimus

The length of preinfection treatment significantly and gradually increased the number of plaques, depending on the length of the everolimus treatment, compared with the untreated control infected on the same day, as shown in Fig. 2C. Because everolimus treatment was not saturated in five days, this indicated that it was necessary for the cells to exhibit the maximal efficacy of everolimus after at least five days, and that everolimus would gradually change the cellular properties and modify the susceptibility to CMV infection. Thus, the effect of everolimus on CMV should be assessed by treating cells for a certain period of time before infection because various cellular factors may contribute to CMV infection, indicating the complexity of cellular factors modified in a time-dependent manner by everolimus.

3.4. Effect of everolimus on virus adsorption and early phase viral protein synthesis

Virus adsorption was evaluated in cells pretreated for 24 h with 5 ng/mL everolimus and infected at 2 PFU/cell, and the viral DNA in the cells were extracted at 24 h after infection (Fig. 3). Everolimus significantly reduced CMV DNA amounts in everolimus-treated cells compared to untreated cells (37.8% in Fig. 3A and 73.6% in Fig. 3B) ($P < 0.01$). Thus, the virus adsorption step was impaired in everolimus-treated cells.

3.5. Everolimus suppressed spread of CMV infection and reduced the number of infected cells

Because the size of the plaques was smaller in everolimus-treated cells than in cells that received no treatment, the spread of infection was evaluated by the number of infected cells in CMV-infected cultures with or without treatment with 5 ng/mL everolimus. Fig. 4 shows the time-course comparison of the numbers of infected cells in the cultures with and without everolimus. The number of infected cells significantly increased by 2.26 and 2.30 times in everolimus-treated cells compared to untreated cells ($P < 0.0001$), whereas the number did not change

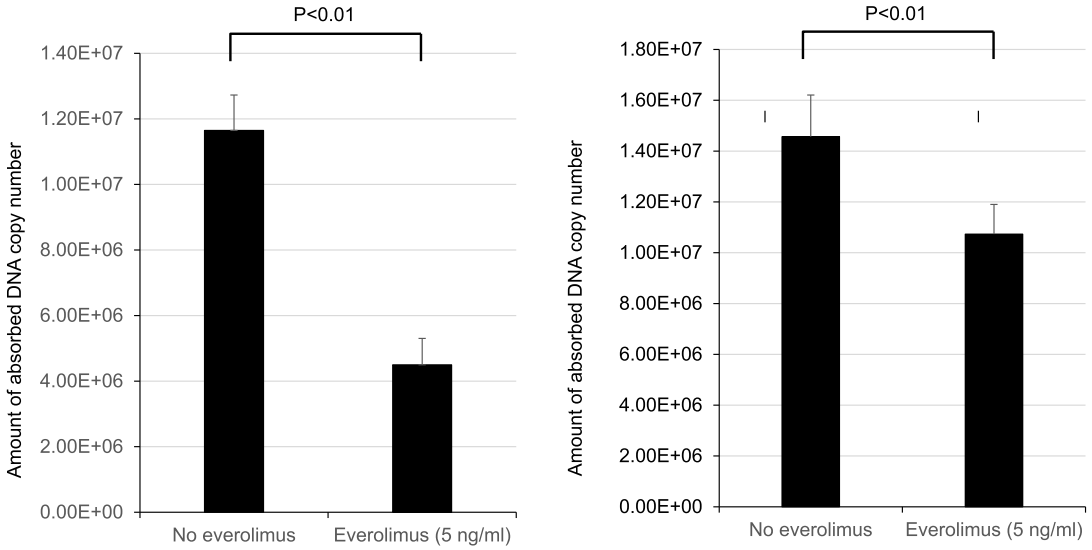

Fig. 3. Effect of virus adsorption and penetration of CMV in everolimus-treated cells. Cells in 12 wells were pretreated with and without 5 ng/mL everolimus, and then infected with 2 PFU/cell for 1 h. The cells were washed three times, incubated for 24 h without everolimus, and washed three times with medium. The cells were lysed, and the amount of CMV DNA was quantitated by real-time PCR. Cellular DNA was suspended in 50 μL of elution buffer, and the number of CMV DNA copies in 2 μL was determined and expressed as the DNA copy number. The amount of absorbed DNA was expressed as the mean ± SD (n = 4) in two experiments, and comparison was made by Student's *t* test.

from day 0–1 and 2 days in the infected cultures without everolimus because spread of infection was observed 3 days after infection. This increase in the number of infected cells in the initial phase by everolimus was consistent with the increase in the plaque number. Interestingly, the total number of infected cells was approximately twice as high in the cultures without everolimus than in those with everolimus later than day 5 and up to day 9. Everolimus treatment suppressed the spread of infection to neighbouring cells and reduced the number of infected cells in the everolimus-treated culture on day 9–39.3% and 43.2% of those without everolimus ($P < 0.0001$). These results indicate that everolimus suppressed the spread of infection to 40% in untreated cultures and resulted in smaller plaques in spite of the increased number of infected cells in the initial phase.

3.6. Effects of everolimus on viral DNA synthesis and viral DNA release in CMV-infected cultures

Fig. 5 shows the comparison of viral DNA synthesis (A) and viral DNA release in CMV-infected cultures (B) treated with and without 5 ng/mL everolimus. Everolimus treatment significantly delayed and reduced viral DNA synthesis in the cells and viral DNA release into the culture supernatant by the repeated-measures ANOVA ($P < 0.0001$).

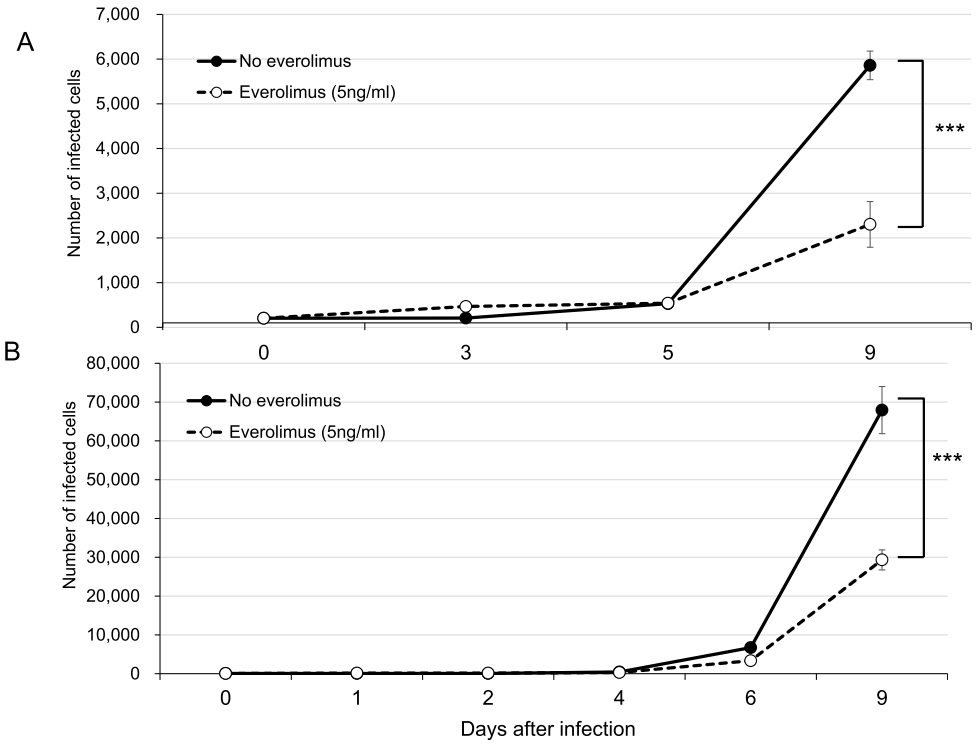

Fig. 4. Time course of the spread of infection in infected cultures treated with and without 5 ng/mL of everolimus. The cultures were harvested each day for determination of the number of infected cells by the infectious centre assay. The numbers of infected cells were shown as the mean ± standard deviation (n = 6) in two experiments. The mean numbers of infected cells treated with and without 5 ng/mL of everolimus were 206.7 and 466.7 (226% of untreated cultures) on day 3 and 5860 and 2303 (39.3% of untreated cultures) on day 9 in Fig. 2A ($P < 0.0001$ by t test). The mean numbers of infected cells treated with and without 5 ng/mL of everolimus were 66.2 and 152.4 (230% of untreated cultures) on day 2 and 67,933 and 29,333 (43.2% of untreated cultures) on day 9 ($P < 0.0001$ by Student's *t* test) in Fig. 2B. ***, a significant difference ($P < 0.0001$) in the cultures between control (no everolimus) and 5 ng/mL everolimus. Thus, the number of infected cells was reduced to approximately 40% in everolimus-treated cultures compared to untreated cultures on day 9.

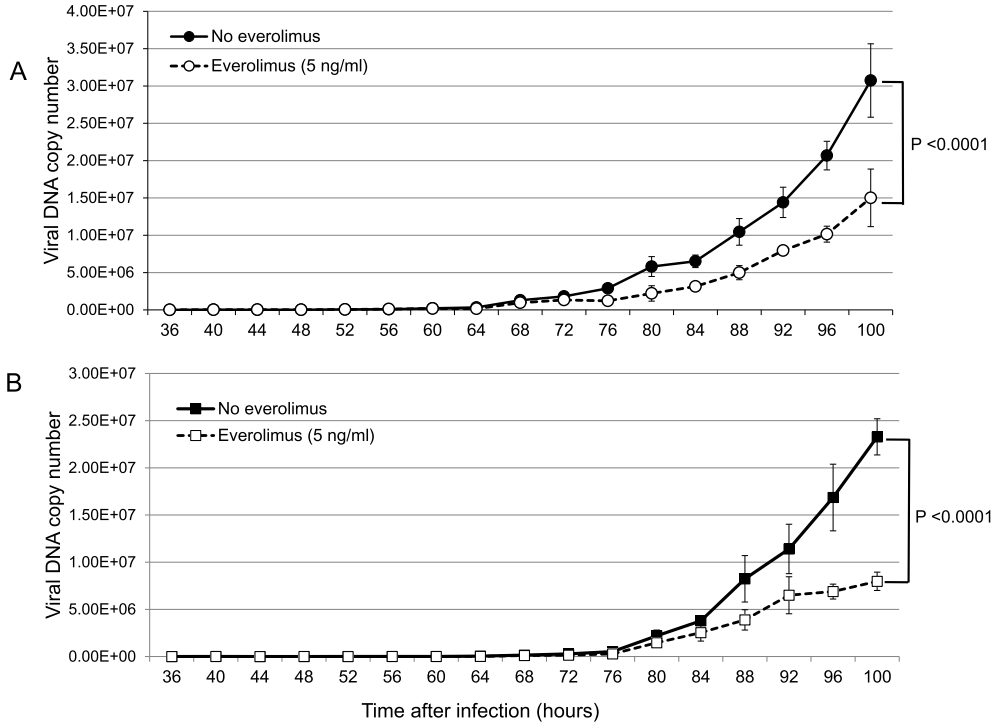

Fig. 5. Cells in 48-well dishes treated with and without 5 ng/mL of everolimus for 24 h, infected with 2 PFU/cell in quadruplicate, and incubated for the indicated time in medium with and without 5 ng/mL everolimus. The culture supernatants and cellular fractions from four wells for each treatment were collected at the indicated time, and CMV DNA was quantified by real-time PCR. Cellular and supernatant DNA was suspended in 50 µL of elution buffer, and the number CMV DNA copies in 2 µL was determined and expressed as the DNA copy number. Panels A and B show the amounts of CMV DNA in the cellular and supernatant fractions, respectively, with (as everolimus) and without everolimus treatment (No everolimus, as the control). DNA copy numbers are expressed as the mean ± SD (n = 4). The difference in the number of CMV DNA copies between no everolimus and everolimus treatment was analysed by repeated-measures ANOVA and was significantly different in the viral DNA copy number in the both cellular fraction and culture supernatants, with values of $P < 0.0001$, as shown in the figure.

Viral DNA synthesis in infected cells without everolimus was observed from 68 h, and viral DNA release into the culture medium occurred 80 h after infection. DNA synthesis was delayed approximately 8 h, and a subsequent delay in viral DNA release was observed in everolimus-treated cultures. The reduction in everolimus-treated cultures was 48.9% and 34.2% of untreated cultures in viral DNA synthesis and viral DNA release, respectively, at 100 h after infection. Pre- and postinfection treatments with everolimus clearly caused a reduction and delay in viral DNA synthesis, followed by a reduction and delay in the release of viral DNA and possibly viral particles into the culture supernatant, and thereby the spread of CMV infection to neighbouring cells was reduced and delayed, resulting in a reduction of the spread of CMV infection and smaller CMV lesions.

4. Discussion

Because everolimus alleviates CMV infection in transplant recipients, we first tried to characterise the anti-CMV action without pretreatment, as other researchers have done (Kudchodkar et al., 2004, 2007; Moorman and Shenk, 2010). Although CMV replication was not significantly affected by various concentrations of everolimus without preinfection treatment, we found that preinfection treatment and its length were important to demonstrate the suppressing activity of everolimus on spreading CMV infection. Everolimus reduced virus adsorption and the spread of CMV infection, and it reduced and delayed viral DNA synthesis in the cells and the release of viral DNA from cells to the culture supernatant. Everolimus treatment of the cells caused formation of smaller plaques and a reduction to approximately 40% in the total number of infected cells in the CMV-infected culture on day 9, corresponding to two to three replication cycles. The average plaque size was reduced from 0.11 mm^2 to 0.04 mm^2 (36.4%), and this was similar to the total number of infected cells assessed by the infectious centre assay. This reduction in the total number of infected cells to 40% in the plate (two dimensions) on day 9 corresponded to $0.4 \times 0.4 = 0.16$ in the solid (three dimensions), indicating that the volume of CMV lesions was reduced to one-sixth by everolimus. Low and delayed CMV replication was supported by the delay and reduction in viral DNA synthesis and viral release in everolimus-treated cultures. The mechanisms of this everolimus anti-CMV action may be complex processes, and many cellular factors might contribute to anti-CMV activity. CMV replication is highly dependent on cellular factors, and everolimus modifies many cellular transcriptional and translational processes related to CMV replication. The target of everolimus, mTORC1, regulates translation and cell growth, and the importance of pretreatment of the cells indicated that many factors might contribute to the observed anti-CMV activity. The mechanism of the suppression of CMV is not clearly understood, although extensive studies on the action of everolimus on CMV have been performed (Clippinger et al., 2011a, b; Kudchodkar et al., 2007; Kudchodkar et al., 2004; Moorman and Shenk, 2010; Roy and Arav-Boger, 2014).

Everolimus treatment showed an increase in the number of plaques and a reduction in plaque size, depending on concentration and treatment time (Figs. 1 and 2). Everolimus-pretreated cells adsorbed less virus with more plaque formation in the initial phase of infection (Fig. 3). The ability and efficiency of infection by virus particles depends on the attachment to cellular receptors, uncoating, transport to the nucleus, and viral factors to establish infection. Infectivity per DNA copy number was 1.65 ± 1.22 PFU/1000 DNA copies in our four CMV stocks, and one infectious virus would be supported by many abortive viruses. Everolimus might modify the cells to be approximately 50% less susceptible to virus particles but support may be five times stronger for the cellular events required for establishing infection, resulting in an increase in plaque number but reduced and delayed DNA synthesis. Delayed and reduced viral DNA synthesis by everolimus may contribute to the reduced spread and small plaque formation in the everolimus-treated cultures.

CMV replication is highly dependent on beneficial host cell translation functions. As a major regulator of cap-dependent translation initiation, the activation of mTOR is essential for CMV. Activated mTOR can phosphorylate 4E-BP, eIF4G, and S6K. Further, phosphorylation of 4E-BP and eIF4G can maintain the formation and integrity of the eIf4F complex, and thus maintain efficient translation (Clippinger et al., 2011a, b; Kudchodkar et al., 2007; Kudchodkar et al., 2004; Clippinger and Alwine, 2012). HCMV infection and the major HCMV immediate-

early proteins (MIEPs) can active mTOR by activating P13K/Akt signalling, to maintain the eIF4F complex and translation (Kudchodkar et al., 2004). With respect to mTOR's role in translational control, HCMV depends on it early in infection but can bypass it later in infection (Clippinger and Alwine, 2012). It may be the reason why everolimus delayed HCMV DNA synthesis. It has been reported that HCMV induces mTOR-independent mechanisms for the phosphorylation of 4E-BP and eIF4G (Clippinger and Alwine, 2012; Clippinger et al., 2011a, b; Kudchodkar et al., 2004).

It seems reasonable that everolimus, an mTOR inhibitor, can inhibit CMV, but many studies shown that everolimus inhibits CMV only to a modest extent. The reason is HCMV can circumvent the inhibition of mTOR and maintenance of mTORC1 activity by coocalization of mTOR and Ras homology enriched in brain protein-GTP in the perinuclear compartment by everolimus (Clippinger et al., 2011b). Thus it seems difficult to properly reconcile previous reports and the results of this study.

The longer preinfection treatment with everolimus increased plaque formation with reduced plaque size, and modification of the properties of the cell over a long period of time seems to be the effect of everolimus when it is involved in the treatment of cancer cells. It was revealed by changes in the properties of cells over time that everolimus helped alleviate CMV infection in transplant patients.

5. Conclusion

Pretreatment was essential to understand the effect of everolimus on CMV infection. The major effect of everolimus on CMV was a reduction in viral adsorption and a reduction and delay of CMV DNA synthesis and virus release into the culture supernatant, and these anti-CMV actions resulted in formation of small plaques corresponding to at least one-sixth the size of CMV lesions in two to three replication cycles. Thus, sensitization of the cells with everolimus during a certain period is important for reducing adsorption and replication, leading to limited CMV lesions, resulting in alleviation of CMV infection in transplant recipients.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.12.004.

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