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Zinc accelerates dengue virus type 2-induced apoptosis in Vero cells

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Abstract Dengue virus type 2 (DENV-2) infection induced apoptotic cellular DNA fragmentation in Vero cells within 8 days of infection. The addition of high concentrations of extracellular Zn^{2+} but not Ca^{2+}, Mg^{2+} or Mn^{2+} to the cell culture medium hastened the detection of apoptosis to within 4 h after infection. No apoptotic cellular DNA fragmentation was detected in the cell culture treated with Zn^{2+} alone or infected with heat- or ultraviolet light-inactivated DENV-2 in the presence of Zn^{2+}. These results suggest that (i) apoptosis is induced in African green monkey kidney cells infected with live DENV-2 and (ii) the addition of high extracellular Zn^{2+} accelerates detection of apoptosis in the DENV-2-infected cells. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Apoptosis; Cation; Dengue; Vero; Zinc

1. Introduction

Dengue virus, the causal agent of dengue, has been shown to induce apoptosis in vitro and in vivo [1–6]. The mechanisms that trigger the apoptotic cellular responses, however, have not been thoroughly investigated. Despres et al. [1] suggested that accumulation of viral proteins in the endoplasmic reticulum (ER) leads to apoptotic cell death by activation of the ER stress-induced apoptotic pathway. This pathway has been suggested to be associated with increased intracellular Ca^{2+} levels that promote DNA degradation by activating the Ca^{2+}/Mg^{2+}-dependent endonucleases [7–10]. In a recent study, Jan et al. [11] suggested that the release of arachidonic acid (AA) by dengue virus-activated phospholipase A_2 (PLA_2) stimulates the production of superoxide anions and activates nuclear factor κB (NF-κB), leading to the induction of apoptosis. They proposed that activation of PLA_2 by events occurring at the cell membrane during the very early stages of infection, prior to viral protein synthesis, is sufficient to trigger the apoptotic signaling pathways. This is in contrast to the earlier suggestion by Despres et al. [1] that accumulation of viral proteins was necessary. Regardless of the initial triggering mechanisms, both studies agreed that the NF-κB is eventually activated. Since both pathways have been shown in other systems to be influenced by divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}, it is suggested that perhaps induction of apoptosis by dengue viruses is also affected by these ions. In the present investigation, the potential effects of extracellular addition of selected divalent cations, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}, on dengue virus-induced apoptosis in African green monkey kidney cells were examined.

2. Materials and methods

2.1. Cells and virus inoculum

Cells and virus inoculum used in the study were prepared as previously described [12]. Briefly, the C6/36 mosquito (*Aedes albopictus*) and Vero cells, purchased from the American Type Culture Collection (ATCC, USA), were cultured in Eagle's minimum essential medium (Gibco BRL, USA) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Austria). The virus inoculum was prepared by infecting the C6/36 cell cultures at 70–80% confluency with dengue virus type 2 (DENV-2) (New Guinea C strain) to give an estimated multiplicity of infection (m.o.i.) of about 0.01 plaque forming unit per cell (p.f.u./cell). Once the infected cell cultures showed about 90% cytopathic effects, the infected cells were frozen and stored at −70°C. The virus inoculum was then prepared by freeze–thawing the infected cell cultures and sedimenting the supernatant at $40\,000 \times g$ to remove the cell debris. The cell culture supernatant obtained from the centrifugation was used as the virus inoculum. The infectivity of the inoculum was determined by performing viral plaque assays using porcine spleen cells [13]. In experiments where ultraviolet light (UV)-inactivated virus was needed, dengue virus inoculum was irradiated with UV at 312 nm on an ice bed using Spectrolinker XL-1500 UV Crosslinker (Spectronics, USA). The intensity of the UV dose applied was measured using a radiometer (Spectronics). For heat inactivation, virus inoculum placed in a microcentrifuge tube was incubated in a 56°C water bath for 30 min or boiled for 10 min. After the treatment, the inoculum was immediately placed on ice and the infectivity was determined by performing virus plaque assays.

2.2. Virus infection and treatment of cells with divalent cations

Throughout the study, unless otherwise mentioned, Vero cells cultured in 25 cm^2 tissue culture flasks (Corning, USA) were infected with virus inoculum to give an estimated m.o.i. of about 0.5 p.f.u./cell, or mock-infected using uninfected C6/36 cell lysate prepared in parallel with the virus inoculum. The infected cell cultures were incubated at 37°C for 1 h with gentle shaking at 10 min intervals. After the incubation, serum free medium containing selected concentrations (0.1, 0.5, 1.0, 2.5 and 5.0 mM) of $CaCl_2$ (Ca^{2+}), $MgSO_4$ (Mg^{2+}), $MnCl_2$ (Mn^{2+}) or $ZnSO_4$ (Zn^{2+}) was added to the cell cultures. FCS was then added to give a final concentration of 5% and the pH of the growth medium was maintained at 7.2–7.4 using 7.5% sodium bicarbonate in phosphate-buffered saline (PBS; 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 M NaCl, 2.7 mM KCl). Traces of divalent cations present in the tissue culture medium and the serum were not considered when calculating the concentrations of the added cations since equal amounts of these ions were present in both the treated and the mock-treated controls. The tissue culture flasks were then incubated at 37°C until cells were harvested.

2.3. Preparation of cellular DNA for electrophoresis

At selected intervals after infection, infected and mock-infected cells

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Abbreviations: DENV-2, dengue virus type 2; m.o.i., multiplicity of infection; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

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were scraped into the growth medium and then sedimented by centrifugation at 800×*g*. The cell pellets were rinsed once in PBS and lysed in lysis solution consisting of 1% *N*-lauroylsarcosine, 0.2% sodium deoxycholate, and proteinase K (1 mg/ml; Sigma, USA) prepared in L-buffer (0.01 M Tris–HCl, pH 7.6, 0.02 M NaCl, 0.1 M EDTA) at 37°C and left overnight. Following the incubation and heat inactivation of the proteinase K (50°C for 1 h), RNase A (1 mg/ml; Sigma, USA) was added and the samples were incubated for an additional 4 h at 37°C. DNA was quantitated using the GeneQuant DNA/RNA calculator (Amersham Biosciences, Sweden) and kept at 4°C. An equal amount of DNA (~4 μg) from each sample was then electrophoresed in 1.8% agarose gel at 80 V for 1 h.

2.4. Detection of DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)

Apoptotic cells were detected using a TUNEL system (Promega, USA) following the protocol provided by the manufacturer. Cells were counterstained with propidium iodide (Sigma, USA) or stained for detection of DENV-2 antigen using mouse hyperimmune sera against DENV-2 and TRITC-conjugated goat anti-mouse IgG (Sigma, USA). The doubly stained samples were viewed under a confocal microscope (Bio-Rad MRC-1024; Bio-Rad, USA) and images were captured and digitized. The percentage of apoptotic and antigen-positive cells was calculated by counting the number of cells in the printed image of a microscope field at 400× magnification and dividing it by the total number of cells observed per field. At least 10 randomly picked microscope fields were used for determining the mean ± standard deviation (S.D.) of the percentages per datum. Statistical analyses using analysis of variance (ANOVA) and Pearson correlation were performed using the SPSS for Windows version 10.5A (SPSS, USA).

3. Results

3.1. Induction of apoptosis in Vero cells

Apoptotic cellular DNA fragments were detected by agarose gel electrophoresis in DENV-2-infected Vero cells after 8 days of infection (Fig. 1a). No cellular DNA fragmentation, however, was noted in the mock-treated samples prepared in parallel. Using TUNEL which labeled the 5′ end of the fragmented DNA, approximately 7.1% of the infected cells showed evidence of cellular DNA fragmentation after 5 days of infection (Fig. 1b,c). The percentage of TUNEL-positive cells increased to ~22.0 and 30.0% at 8 and 11 days after infection, respectively (Fig. 1c). The percentage of DENV-2 antigen-positive cells also increased steadily, beginning on day 3 after infection and by day 11, approximately 96.8% of the infected cells were DENV-2-positive. A significant correlation ($r > 0.75$, Pearson) between the presence of DENV-2 antigen and TUNEL-positive cells was observed. The percentage of cells positive with TUNEL after 8 days of infection, however, diminished significantly ($P < 0.001$, ANOVA) with decreasing m.o.i. used (Fig. 2). At m.o.i. of 0.005 p.f.u./cell, for example, only approximately 9.0% of the infected cells were apoptotic. Furthermore, no (<1%) TUNEL-positive cells were noted when heat-treated or boiled inoculum was used.

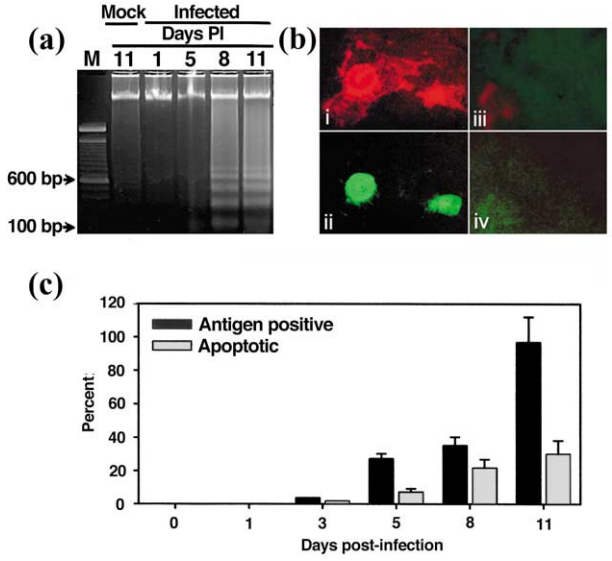

Fig. 1. Induction of apoptosis in Vero cells by DENV-2. Oligonucleosomal DNA fragments, characteristic of apoptotic cells, were detected following agarose gel electrophoresis (a), confocal microscopy (b) and TUNEL staining (c). DENV-2-positive cells (b, i) prepared 8 days after infection were also positive with TUNEL (b, ii). Mock-infected cells similarly prepared, however, were negative when stained for DENV-2 antigen (b, iii) and TUNEL (b, iv). The percentage of apoptotic cells increased steadily with the increasing number of DENV-2 antigen-positive cells (c). The percentages of TUNEL and DENV-2 antigen-positive cells were determined as described in Section 2. Results shown in (c) are mean ± S.D. of the percentages. M shows the DNA size markers.

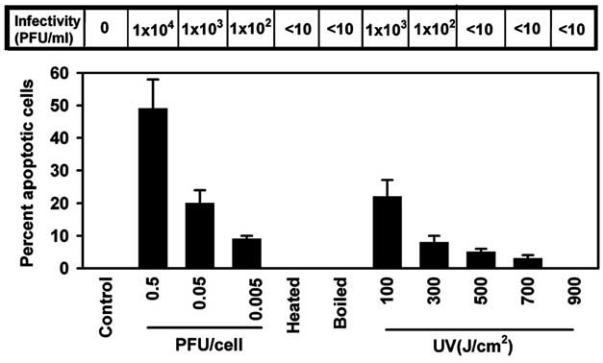

Fig. 2. The influence of viral infectivity on DENV-2-induced apoptosis. DENV-2-infected cells were stained with TUNEL and then counterstained with propidium iodide on day 8 after infection. The number of TUNEL-positive cells was determined and the percentage over the total number of cells was calculated. Results are presented as mean ± S.D. of the percentages as described in Section 2.

Potential dependency on infectious virus particles was demonstrated by using dengue virus inoculum that had been inactivated with selected dosages of UV irradiation. A significant dose-dependent reduction in the number of apoptotic cells was observed with increased UV irradiation (Fig. 2) of the virus inoculum. Reduction by approximately 50 and 80% of TUNEL-positive cells was obtained using virus inoculum treated with 100 and 300 J/cm^2 of UV light, respectively. No (<1%) TUNEL-positive cells were noted when the cells were infected with virus inoculum treated with UV light at 900 J/cm^2 or higher. At these intensities, the UV-inactivated virus retained the ability to bind to cells (data not shown) but infectious virus was not detectable by virus plaque assays.

3.2. The effects of divalent cations on DENV-2-induced apoptosis

The effects of adding Ca^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+} to Vero cells infected with DENV-2 at an estimated m.o.i. of 0.5 p.f.u./cell were subsequently examined. Initial findings showed that after 48 h of infection no cellular morphological changes were noted in the infected or mock-infected cells treated with

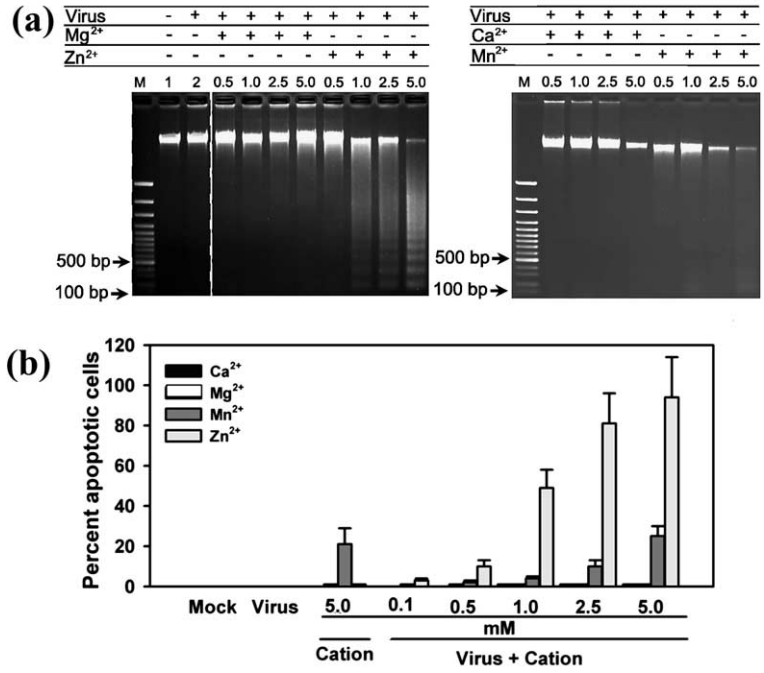

Fig. 3. The effects of divalent cations on DENV-2-induced apoptosis. Infected cells were treated with Ca^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+} and harvested at 48 h after infection. Apoptotic oligonucleosomal DNA fragments were detected following agarose gel electrophoresis (a) and TUNEL staining (b). The percentage of TUNEL-positive cells was calculated as described in Section 2. Results are shown as mean ± S.D. of the percentages obtained from at least 10 replicates. DNA size markers are shown in lane M.

Ca^{2+} and Mg^{2+} at concentrations ranging from 0.1 to 5.0 mM. The treatments also had no observable effects on DENV-2-induced DNA fragmentation (Fig. 3a). Additions of Mn^{2+} or Zn^{2+} at 1.0, 2.5 and 5.0 mM, however, significantly altered the cells' morphology, causing almost 90% of the treated cells to detach from the tissue culture flasks' surfaces. The treated cells also showed an increase in a dose-dependent manner in the oligonucleosomal DNA fragmentation (Fig. 3a). Using TUNEL, approximately 25.0% of the mock-infected cells treated with Mn^{2+} were positive (Fig. 3b), thereby suggesting that high Mn^{2+} concentrations alone could trigger apoptosis. In contrast, no (< 1%) TUNEL-positive cells were noted in the mock-infected cells treated with Zn^{2+} alone. In DENV-2-infected cells, however, the addition of Zn^{2+} at 1.0, 2.5 and 5.0 mM increased the number of apoptotic cells to approximately 49.0, 81.0 and 94.0%, respectively (Fig. 3b).

3.3. The influence of Zn^{2+} on DENV-2-induced apoptosis

Apoptotic oligonucleosomal DNA fragments were detected by agarose gel electrophoresis in DENV-2-infected cells treated with 1.0 mM Zn^{2+} within 12 h of infection (Fig. 4a). These DNA fragments were present only in the Zn^{2+}-treated DENV-2-infected Vero cells but not in the mock-infected controls or Zn^{2+} treatment alone. High molecular mass DNA (Fig. 4a, arrowhead) remained in the mock-infected samples untreated or treated with Zn^{2+} alone and in the untreated DENV-2-infected cells. By 12 h of infection, however, the presence of this high molecular mass DNA in the Zn^{2+}-treated DENV-2-infected cells diminished. This was reciprocated with the appearance of the oligonucleosomal DNA fragments, suggesting that the high molecular mass DNA could have undergone substantial endonuclease digestion. Intense nuclear fluorescence staining of the DENV-2-infected cells treated with 1.0 mM Zn^{2+} was detected within 4 h of infection using TUNEL (Fig. 4b, iv). The staining intensity increased, gradually reaching maximum intensity after about 12 h of infection (Fig. 4b, vi). This observation concurred with the earlier findings obtained using agarose gel electrophoresis, which showed that after 12 h of infection, most of the high

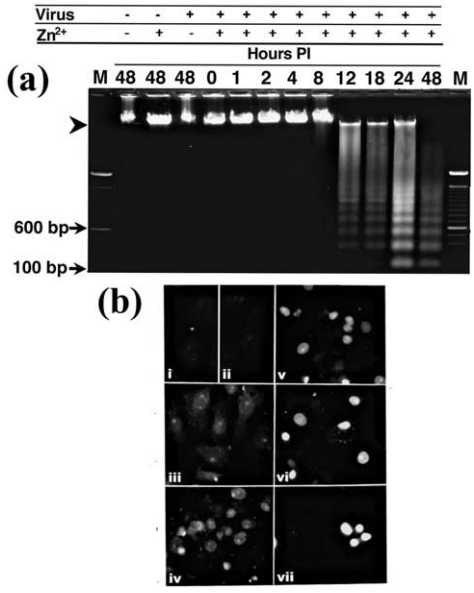

Fig. 4. Time course effects of Zn^{2+} on DENV-2-induced apoptosis. Infected or mock-infected Vero cells were treated with 1.0 mM Zn^{2+}. Cellular DNA was prepared from these cells and separated by agarose gel electrophoresis (a). Concurrently treated cells were also stained with TUNEL (b) at 2 (iii), 4 (iv), 8 (v), 12 (vi) and 24 (vii) h after infection. Control cells infected but not treated with Zn^{2+} or mock-infected but Zn^{2+}-treated prepared at 48 h after infection are as shown in (i) and (ii), respectively. Lane M shows the DNA size markers.

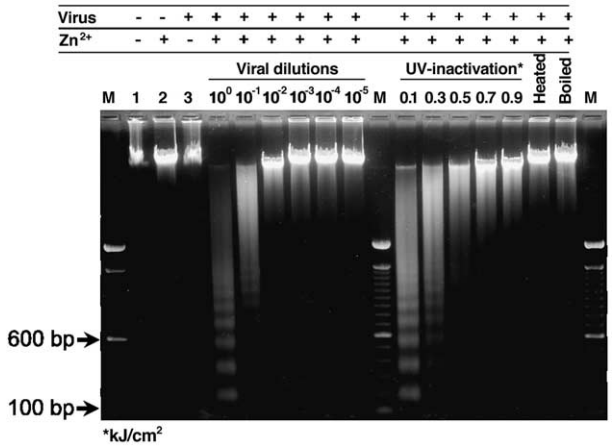

Fig. 5. The influence of viral infectivity on Zn^{2+}-treated DENV-2-induced apoptosis. Vero cells were infected with virus inoculum to give an estimated m.o.i. (determined by plaque assay) of 0.5 (10^{-0}), 0.05 (10^{-1}), 0.005 (10^{-2}), 0.0005 (10^{-3}), 0.00005 (10^{-4}) and 0.000005 (10^{-5}) p.f.u./cell, respectively, or with UV- or heat-inactivated virus with an initial m.o.i. of 0.5 p.f.u./cell in the presence of 1.0 mM Zn^{2+}. DNA samples including the controls (lanes 1–3) were prepared at 48 h after infection. UV inactivation dosages are shown in kJ/cm^2. Heat inactivation was performed at 56°C or boiled at 100°C. M indicates the DNA size markers.

molecular mass DNA has been fragmented into oligonucleosomal DNA fragments.

3.4. The influence of infectious virus particles

Apoptotic cellular DNA fragmentation was detected after 12 h of infection only when DENV-2-infected cells were treated with 1.0 mM Zn^{2+}. Since Zn^{2+} alone did not cause cellular DNA fragmentation, it was suggested that DENV-2 could directly cause the initial DNA damage. Infection of cells with DENV-2 at an input m.o.i. of at least 0.05 p.f.u./cell, however, was necessary for fragmented cellular DNA to be detected by agarose gel electrophoresis (Fig. 5). Similar to the earlier findings, no oligonucleosomal DNA fragments were present when boiled or heat-inactivated DENV-2 inoculum at a m.o.i. of $\sim$0.5 p.f.u./cell (determined prior to inactivation) was used for infection in the presence of Zn^{2+}. UV-inactivated virus inoculum when used with 1.0 mM Zn^{2+} also showed a dose-dependent reduction in the presence of oligonucleosomal DNA fragments with an increase in intensity of UV irradiation used to inactivate the inoculum (Fig. 5). At 500 J/cm^2, when infectious dengue virus was no longer detectable (data not shown), no apoptotic cellular DNA fragments were detected.

4. Discussion

DENV-2 infection of Vero cells resulted in cellular DNA fragmentation characteristic of apoptotic cells. This finding was similar to a number of other previously reported findings using various human [2,4,11,14–16] and non-human cell lines [1,17,18]. Considering these findings and findings presented in the present study, it is apparent that induction of apoptosis is an important cellular event in the pathogenesis of dengue. At low m.o.i. ($<$0.5 p.f.u./cell), which perhaps mimics the natural infection, apoptotic cells were detected in Vero cell cultures after 8 days of infection. A significant correlation between the presence of DENV-2 antigens and apoptotic cells was observed, suggesting that expression of DENV-2 proteins was necessary for apoptosis to occur. This was further supported by the findings that apoptosis occurred in Vero cells only when sufficient numbers of infectious virus particles were used. At very high m.o.i. ($>$10 p.f.u./cell), however, DENV-2 particles could be cytotoxic, resulting in induction of necrosis as previously noted [1].

While the exact mechanisms of DENV-2-induced apoptosis are still being investigated, intracellular accumulation of DENV-2 proteins in the ER was thought to result in induction of intracellular stress which in turn activated the cysteine and serine protease-independent apoptotic pathways [1]. In a separate study, Jan et al. [11] further showed the potential importance of activation of PLA_2 and generation of AA and superoxide anions in DENV-2-induced apoptosis. In their study, it was also reported that treatments of DENV-2-infected cells with $ZnSO_4$ at low concentrations ($<$20 μM) resulted in dose-dependent protection of the infected cells, whereas at higher concentrations, Zn^{2+} became toxic. Similar results, however, were not noted in DENV-2-infected Vero cells treated with low concentrations of Zn^{2+}, suggesting that the effects of low Zn^{2+} concentrations on Vero cells were perhaps different from that of the human neuroblastoma cells used by Jan et al. [11]. This was not surprising, since the SK-N-SH neuroblastoma cells [11] also responded differently to DENV-2 infection when compared to infection of HepG2 hepatoma cells [2]. Nonetheless, in agreement with Jan et al. [11], substantial cellular morphological changes were noted in Vero cells treated with high concentrations of Zn^{2+}. It was further noted in the present study that there was no significant difference between the morphology of cells treated with Zn^{2+} alone and cells infected with DENV-2 and treated with Zn^{2+}. However, when the cellular DNA of these cells was compared, apoptotic cellular DNA fragmentation was noted only in the Zn^{2+}-treated DENV-2-infected cells and not when the cells were treated with either Zn^{2+} or virus alone. Similar results were not observed with cells treated with other divalent cations except Mn^{2+}, where a significant presence of apoptotic cells was noted in both Mn^{2+} treatment alone and in DENV-2-infected cells treated with Mn^{2+}, suggesting that Mn^{2+} alone was inducing the apoptosis. In DENV-2-infected cells treated with Zn^{2+}, induction of apoptosis became detectable within 4 h of infection. This was in contrast to the 8 days of infection when the cells were infected in the absence of Zn^{2+}. Since apoptosis was induced only by infectious DENV-2, it was unlikely that activation of PLA_2 alone, as suggested by Jan et al. [11], could be sufficient to activate the apoptosis signaling pathways. It is thus suggested here that the capacity to induce apoptosis was dependent on specific DENV-2 gene expression. Therefore, the accumulation of viral proteins in the ER resulting in intracellular stress [1] could indeed be the mechanism for DENV-2-induced apoptosis. Zn^{2+}, on the other hand, hastened the apoptotic process triggered initially by the DENV-2 virus gene expression, perhaps by enhancing the DENV-2-induced activation of PLA_2 [19] and the NADPH oxidase [20]. Since the induction of apoptosis has been argued as one of the potential mechanisms whereby the host limits virus infection [21], the acceleration of apoptosis by Zn^{2+} or drugs that mimic the effects of Zn^{2+} could perhaps be exploited to hasten the removal of dengue virus-infected cells, thus preventing further infection.

In summary, the results presented in the present study sug-

gest that DENV-2 infection of Vero cells resulted in the induction of apoptosis that was hastened in the presence of high extracellular Zn^{2+} concentrations.

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References

[1] Despres, P., Flamand, M., Ceccaldi, P.E. and Deubel, V. (1996) J. Virol. 70, 4090–4096.
[2] Marianneau, P., Cardona, A., Edelman, L., Deubel, V. and Despres, P. (1997) J. Virol. 71, 3244–3249.
[3] Shafee, N. and AbuBakar, S. (1997) Asia Pac. J. Mol. Biol. Biotech. 5, 111–117.
[4] Couvelard, A., Marianneau, P., Bedel, C., Drouet, M.T., Vachon, F., Henin, D. and Deubel, V. (1999) Hum. Pathol. 9, 1106–1110.
[5] Lei, H.Y., Yeh, T.M., Liu, H.S., Lin, Y.S., Chen, S.H. and Liu, C.C. (2001) Biomed. Sci. 8, 377–388.
[6] Huerre, M.R., Lan, N.T., Marianneau, P., Hue, N.B., Khun, H., Hung, N.T., Khen, N.T., Drouet, M.T., Huong, V.T.Q., Ha, D.Q., Buisson, Y. and Deubel, V. (2001) Virchows Arch. 438, 107–115.
[7] Duke, R.C., Chervenak, R. and Cohen, J.J. (1983) Proc. Natl. Acad. Sci. USA 80, 6361–6365.
[8] Giannakis, C., Forbes, I.J. and Zalewski, P.D. (1991) Biochem. Biophys. Res. Commun. 181, 915–920.
[9] Barbieri, D., Troiano, L., Grassilli, E., Agnesisi, C., Cristofalo, E.A., Monto, D., Capri, M., Cossarizza, A. and Franceschi, C. (1992) Biochem. Biophys. Res. Commun. 187, 1256–1261.
[10] Lohmann, R.D. and Beyersmann, D. (1994) Environ. Health Persp. 102, 269–271.
[11] Jan, J.T., Chen, B.H., Ma, S.H., Liu, C.I., Tsai, H.P., Wu, H.C., Jiang, S.Y., Yang, K.D. and Shaio, M.F. (2000) J. Virol. 74, 8680–8691.
[12] AbuBakar, S., Azmi, A., Mohammed-Saad, N., Shafee, N. and Chee, H.Y. (1997) Malays. J. Pathol. 19, 41–51.
[13] Rao, B.L. (1976) Indian J. Med. Res. 64, 1709–1712.
[14] Ho, L.J., Wang, J.J., Shaio, M.F., Kao, C.L., Chang, D.M., Han, S.W. and Lai, J.H. (2001) J. Immunol. 166, 1499–1506.
[15] Marianneau, P., Steffan, A.M., Royer, C., Drouet, M.T., Jaeck, D., Kirn, A. and Deubel, V. (1999) J. Virol. 73, 5201–5206.
[16] Avirutnan, P., Malasit, P., Seliger, B., Bhakdi, S. and Husmann, M. (1998) J. Immunol. 161, 6338–6346.
[17] Liao, C.L., Lin, Y.L., Wu, B.C., Tsao, C.H., Wang, M.C., Liu, C.I., Huang, Y.L., Chen, J.H., Wang, J.P. and Chen, L.K. (2001) J. Virol. 75, 7828–7839.
[18] Despress, P., Frenkiel, M.P., Ceccaldi, P.E., Duarte Dos Santos, C. and Deubel, V. (1998) J. Virol. 72, 823–829.
[19] Lindahl, M. and Tagesson, C. (1996) Inflammation 20, 599–611.
[20] Noh, K.M. and Koh, J.Y. (2000) J. Neurosci. 20, 1–5.
[21] Koyama, A.H., Fukumori, T., Fujita, M., Irie, H. and Adachi, A. (2000) Microbes Infect. 2, 1111–1117.