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BRIEF REPORT

Vimentin is required for dengue virus serotype 2 infection but microtubules are not necessary for this process

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Abstract The present study investigated the effect of microtubules (MTs) and vimentin during dengue virus serotype 2 (DV2) infection. Immunostaining showed that DV2 infection induced MT and vimentin reorganization. Colocalization of DV2 antigens with MTs or vimentin were often observed in ECV304 cells. MT-disrupting agents could enhance DV2 release but did not affect other steps of virus replication. In contrast, disruption of vimentin inhibited DV2 infection. Our results suggest that an MT-dependent mechanism may not be necessary for DV2 infection, and MT disruption may promote DV2 release. However, vimentin is required for DV2 infection.

Dengue virus (DV) belongs to the family *Flaviviridae*, and there are four serotypes (DV1-4). They cause a mild-to-debilitating febrile illness (classical dengue fever, DF) or life-threatening syndrome (dengue haemorrhagic fever/dengue shock syndrome, DHF/DSS). In recent years, the geographical range of DV infection in tropical and subtropical regions of the world has extended, and DHF/DSS is occurring in new areas and with increased incidence [11, 14].

Viruses are obligate intracellular parasites and therefore depend on the host cellular machinery to facilitate entry, replication, transport, and release of progeny virions [5]. The intricate structural system referred to as the cytoskeletal network is composed of actin, microtubules (MTs), intermediate filaments (IFs), their motor protein and other elements [10]. Actin filaments are flexible structures which are organized into a variety of linear bundles and arrays, two-dimensional networks or three-dimensional gels. Myosins mediate translocation along actin filaments. MTs are long, hollow cylinders assembled from heterodimers of α- and β-tubulin and MT-associated proteins (MAPs). Directional movement along MT is mediated by the motor proteins kinesin and cytoplasmic dynein [4, 17]. IFs with a diameter of 8–10 nm are found in nearly all eukaryotic cells and show a characteristic tripartite domain organization. Unlike actin and MTs, IFs do not participate in cell motility.

It has been reported that many viruses interact with cytoskeletal elements, which is considered to be critical at each step along the replication cycle. Vaccinia virus requires MTs and cytoplasmic dynein for efficient transport in HeLa cells. MT depolymerizing agents significantly reduce viral capsid accumulation at perinuclear sites [15]. Respiratory syncytial virus replication requires actin [10], and IF integrity is required for Junin virus replication [3]. However, the effect of cytoskeleton in DV2 infection is not fully understood.

Our previous studies demonstrated that actin filaments play an important role in DV2 infection [22]. In the present work, we investigate whether MTs or IFs are required for DV2 infection by immunofluorescence analysis and

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interference of the cytoskeleton with drugs. Our results suggested that an MT-dependent mechanism is not necessary, while vimentin is required for DV2 infection.

The role of MT in DV2 infection

DV2 (strain Tr1751), isolated from a patient with DF, was used in this study. ECV304 cells (European Collection of Cell Cultures), grown on coverslips in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS), were infected with DV2 or heat-inactivated DV2 (56°C for 30 min, mock infection) at a multiplicity of infection (MOI) of one for 1 h at 37°C. Then, cells were cultured with medium in the absence or presence of demecolcine (Sigma), which depolymerizes MTs, at a concentration of 500 ng/ml until 24 h postinfection (p.i.). Immunofluorescence staining was carried out as follows: the cells were fixed with 4% paraformaldehyde for 20 min at 25°C. After washing with phosphate-buffered saline (PBS), the specimens were incubated with 1% bovine serum albumin and then immunostained with rabbit anti-DV2 polyclonal antibody (pAb) and mouse anti-α-tubulin monoclonal antibody (mAb, Sigma, USA) overnight at 4°C. After washing with PBS, the specimens were incubated with TRITC-labeled anti-rabbit antibody (ZSGB-BIO, PRC) and FITC-labeled anti-mouse antibody (Sigma, USA) for 1 h at 37°C and observed in fluorescence microscopy (Olympus, BX51, Japan) or confocal laser scanning microscopy (Leica, TCS-SP2, Germany).

Immunostaining showed that the normal morphology of the MT cytoskeleton in infected ECV304 cells (Fig. 1a) was replaced by aberrant MT forms at 24 h p.i. They could be morphologically classified into three types: (1) a disorganized MT network where MT seems randomly oriented in the cells (Fig. 1b), (2) cells in which MTs formed rings around the nucleus (Fig. 1c), and (3) cells with long projections containing MT bundles (Fig. 1d). Each type of cell was counted and the percentage of the total represented by each type was calculated. A disorganized MT network was observed in about 12%, MT rings in about 8%, and long projections in about 3% of DV2 antigen-positive cells. However, aberrant forms of MT were rarely seen in DV2-infected HepG2 cells (American Type Culture Collection, data not shown), indicating differences in MT reorganization induced by DV2 infection present in the different cell types. Moreover, confocal laser scanning microscopy showed DV2 antigens were highly colocalized with MTs, and they were aligned with long MT bundles (Fig. 1e) or mainly accumulated at the MT organizing center (MTOC, Fig. 1f) and perinuclear region (Fig. 1g). However, colocalization of DV2 antigens with depolymerized tubulin was not observed, and the fluorescence intensity of viral antigens did not show a major change in demecolcine-treated cells (Fig. 1h). The results of reorganization of the MT network induced by DV2 infection and colocalization of DV2 antigens with intact MTs indicated that MTs might be involved in virus infection.

The presence of aberrant MT forms after virus infection was also reported in previous studies. For example, herpes simplex virus type 1 and human cytomegalovirus have been shown to induce disruption of the MT cytoskeleton in infected cells [6, 7]. Vaccinia virus infection can also disrupt MTs. It has been suggested that the MT disruption is probably mediated by the combined effects of viral proteins with MAP-like properties and a loss of the MT-organizing function at the MTOC [15]. In fact, along with changes in the MT cytoskeleton, cell morphology is often drastically affected by virus infection. These alterations are usually referred to as cytopathologic changes, but their extent varies depending on the different virus and cell types [16]. Despite the fact that MT reorganization is a common feature of virus infection, its molecular mechanisms are not readily explained. Based on the present study, we believe that MT reorganization is probably caused by DV2 replication, since disruption of MTs often occurred after

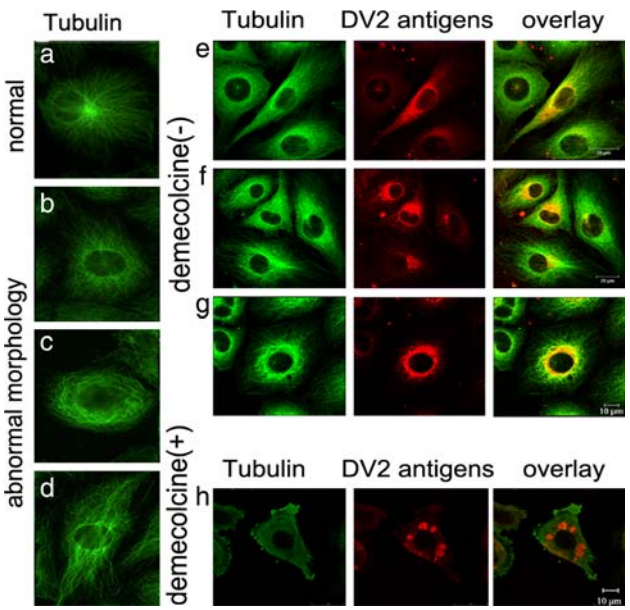

Fig. 1 Immunostaining images showing MT reorganization induced by DV2 infection and colocalization of DV2 antigens (*red*) with MT (*green*) in ECV304 cells at 24 h p.i. **a** Normal pattern of the MT network in mock-infected cells; **b–d** one set of micrographs showing three different morphological forms of the MT cytoskeleton in the infected cells (magnification ×400). **e–g** In untreated cells, viral antigens were aligned with MT bundles or accumulated at the MTOC and perinuclear region. The colocalization of DV2 antigens with tubulin is showed in the merged images; **h** the interaction of DV2 antigens with depolymerized tubulin was rarely observed in demecolcine-treated cells. Bars represent 20 μm (**e**, **f**) and 10 μm (**g**, **h**), respectively

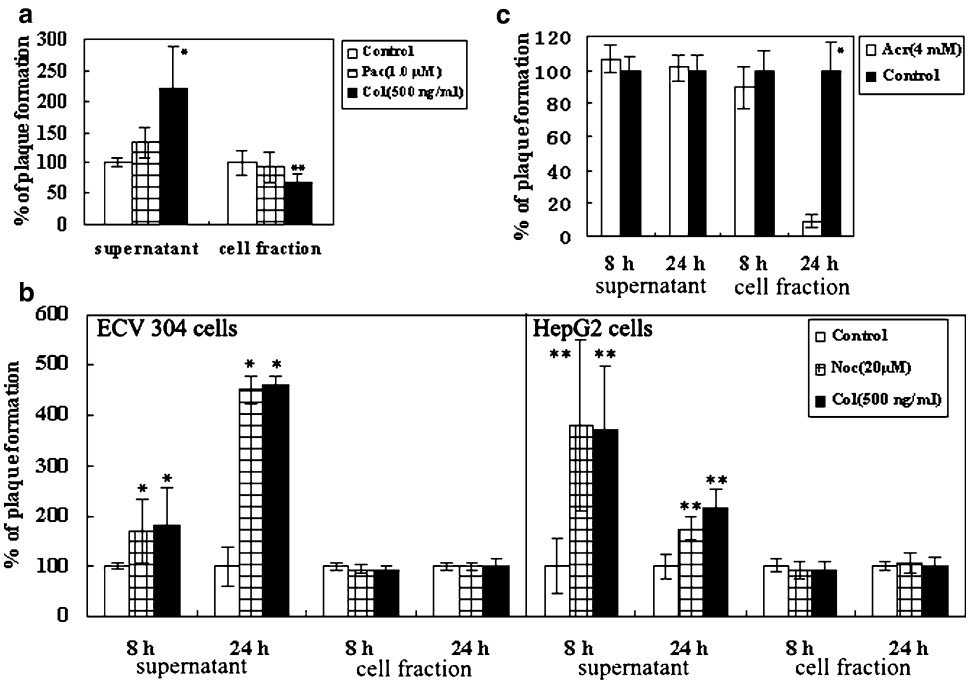

Fig. 2 One set of graphs showing the virus titer by virus plaque assay in different cells during DV2 infection. **a** The disruption of MT could enhance DV2 release. The virus titers of the supernatant and cell fraction were significantly different between the demecolcine-treated group and mock-treated group ($*P < 0.01$ and $**P < 0.05$, respectively). **b** Effect of MT-disrupting drugs on the whole DV2 life cycle. ECV304 and HepG2 cells were pretreated with Col, Noc or 0.1%DMSO (*control*) and then infected with DV2 in the presence of the above drugs. Significant differences in virus titer were observed in the supernatants of both cell lines at 8 and 24 h p.i. ($*P < 0.01$ and $**P < 0.01$, respectively) compared to the virus titer in the supernatant of the mock-treated control. **c** The cells were infected with DV2, and then acrylamide was added and maintained until 8 or 24 h p.i. The effect of acrylamide on DV2 infection in ECV304 cells is shown. No significant difference was seen between the mock- and drug-treated groups in the virus titers of the supernatant and cell fraction at 8 h p.i., but the virus titers of the cell fraction decreased significantly at 24 h p.i. ($*P < 0.01$) compared to the mock-treated control. The values shown are the mean ± SD ($n = 6$). All determinations were performed in three independent experiments. *Col* demecolcine, *Noc* nocodazole, *Pac* paclitaxel, *Acr* acrylamide

extensive accumulation of DV2 antigen in the cytoplasm of ECV304 cells. Further studies will be needed to elucidate the molecular mechanism.

In order to clarify the effect of MTs on the DV2 life cycle, three chemical drugs that interfere with the normal MT depolymerization or polymerization state were used in our experiments. The working concentrations of the drugs demecolcine, nocodazole and paclitaxel were 500 ng/ml, 20 and 1.0 μM, respectively. ECV304 cells were pretreated with each drug or 0.1% dimethyl sulfoxide (DMSO, mock treated) for 1 h at 37°C, respectively, and then infected with DV2 (MOI = 10) for 1 h at 37°C. After removing excess or unbound virus by extensive washing, the cells were cultured in DMEM with 2% FBS at 37°C until 5 h p.i. when the supernatant and cell fraction were collected for detection DV2 entry by plaque assay. Unexpectedly, virus titers of supernatant and cell fraction showed no significant difference between the drug-treated and mock-treated groups at 5 h p.i. (data not shown), indicating that MT seems to have less effect on entry of DV2 into ECV304 cells.

To investigate whether MT participate in DV2 release, ECV304 cells were infected with DV2 (MOI = 1) for 1 h at 37°C. After removing excess or unbound virus, the cells were maintained in DMEM with 2% FBS at 37°C. Based on the DV2 growth curves in ECV304 cells, we determined that the DV2 progeny virus began to be released from the infected cells at 5–8 h p.i. (data not shown). Therefore, demecolcine or paclitaxel were added to the medium at 5 h p.i., and the supernatant and cell fraction were collected at 8 h p.i. As shown in Fig. 2a, the virus titers of both the supernatant and cell fraction showed only slight changes in the paclitaxel-treated group. Interestingly, the virus titer of the supernatant in the demecolcine-treated group was twofold higher than in the mock-treated control ($P < 0.01$, $n = 6$), whereas the virus titer of the cell fraction was reduced to 67% compared to control ($P < 0.05$, $n = 6$), indicating that MT disruption could enhance DV2 release from the infected cells.

Finally, to evaluate the effect of MT disruption on the whole DV2 life cycle, ECV304 were incubated with demecolcine, nocodazole or 0.1% DMSO (mock treated)

for 1 h at 37°C and then infected with DV2 (MOI = 1) for 1 h at 37°C. After adsorption, the medium containing demecolcine, nocodazole or DMSO was added and maintained until 8 or 24 h p.i. In demecolcine/nocodazole-treated cells, the virus titer in the supernatant was twofold and fivefold higher than the mock-treated control at 8 or 24 h p.i., respectively ($P < 0.01$, $n = 6$, Fig. 2b), whereas the virus titer of the cell fraction showed no significant difference between the drug-treated groups and the mock-treated group at the same time point (Fig. 2b). The same experiments were also performed using HepG2 cells, and as expected, a similar result was obtained (Fig. 2b). These data suggest that disruption of MTs might not prevent DV2 from moving and replicating in infected cells.

Our findings are consistent with a report by Salminen et al. who found that depolymerizing the MT with nocodazole speeded up baculovirus gene expression [19]. Recently, some reports have shown that DV particles might possibly be targeted to different transport routes [1, 21], but the mechanism involved in the cell transport process of DV2 is poorly understood. In the present study, DV2 infection could induce MT reorganization, and DV2 antigens colocalized with intact MTs, but treatment with MT-disrupting agents rarely affected DV2 production or replication. Taken together, our results support the possibility that DV2 might interact with intact MTs in infected ECV304 cells, and virus particles might use a microtubule-dependent mechanism for infecting host cells under normal conditions. However, DV2 also could utilize an MT-independent mechanism to achieve successful infection in the absence of intact MTs. In other words, MTs are helpful, but not necessary, for DV2 infection.

The role of vimentin in DV2 infection

IFs are composed of at least 40 different proteins which belong to five different types, types I–V [8]. Since IFs have no polarity and no motor proteins, they are not considered to play any role in intracellular transport [4]. Therefore, only a few experiments on the interactions between IFs and virus infection have been reported. ECV304 cells have a network of IFs mostly made of vimentin. Olink and Sager documented that acrylamide could selectively induce primary collapse and disruption of vimentin structures [13, 18]. Therefore, the colocalization of DV2 antigens with vimentin and the effect of acrylamide on DV2 replication in ECV304 cells were also analyzed in the present study.

ECV304 cells were infected with DV2 (MOI = 1) or heat-inactivated DV2 for 1 h at 37°C and then maintained in the absence or presence of acrylamide (4 mM, BioDev, PRC) until 24 h p.i. Immunofluorescence staining was carried out as follows: The cells were fixed and incubated with rabbit anti-DV2 pAb and mouse anti-vimentin mAb (ZSGB-BIO, PRC) overnight at 4°C. After washing with PBS, the cells were incubated with Cy3-labeled anti-rabbit (Chemicon, USA) and FITC-labeled anti-mouse antibodies to detect DV2 antigens and vimentin, respectively, and observed as described above. Our results showed that vimentin filaments emanated from the perinuclear region and extended into the cytoplasm in mock-infected cells (Fig. 3a), while the filaments retracted from the cell periphery and surrounded the nucleus in DV2-infected cells (Fig. 3b). Meanwhile, DV2 antigens often distributed in the same area (Fig. 3c) and colocalized with vimentin proteins (Fig. 3d), indicating that DV2 infection induced vimentin reorganization in the ECV304 cells. It was noted that the fluorescence intensity of the DV2 antigens became faint, and colocalization with vimentin was not seen after treatment with acrylamide (Fig. 3e), indicating that vimentin reorganization might be involved in the DV2 infection process.

It has been reported that some viruses reorganize the IF network in infected cells during virus replication. For example, an avian strain of influenza A virus induced rearrangement of IFs from an radial pattern into an array surrounding the nucleus [2]. African swine fever virus infection led to rearrangement of vimentin into a cage around virus factories which could prevent the movement of virion cores into the cytoplasm. The virion cores contain enzymes that are necessary for viral genome replication, and their locations are suitable for the production of viral DNA [9, 20]. In addition, it was reported that IFs are intimately associated with MTs in normal cells and that the disruption of MTs by colchicine causes a retraction of IFs to the perinucleus, forming juxtanuclear caps or rings [12]. A similar redistribution of IFs was also seen in DV2 infection, also indicating that vimentin reorganization induced by DV2 infection might be associated with MT disruption.

To further confirm the effect of vimentin on DV2 infection, ECV304 cells were infected with DV2 (MOI = 1). After removing excess or unbound virus by washing, the cells were maintained in DMEM with 2% FBS or in the presence of acrylamide (4 mM) until sample collection. At 8 h p.i., the virus titers of both the supernatant and the cell fraction showed no significant changes compared to untreated controls (Fig. 2c). Interestingly, it was noted that the virus titers of the supernatants in the acrylamide-treated group were not significantly different from that of DV2 infected alone at 24 h p.i., but the virus titers of the cell fractions significantly reduced to 8.91% in the acrylamide-treated group when compared to the control group at the same time point ($P < 0.01$, $n = 6$, Fig. 2c). Moreover, the fluorescence intensity of the DV2 antigens was clearly weak in the cytoplasm, and colocalization of

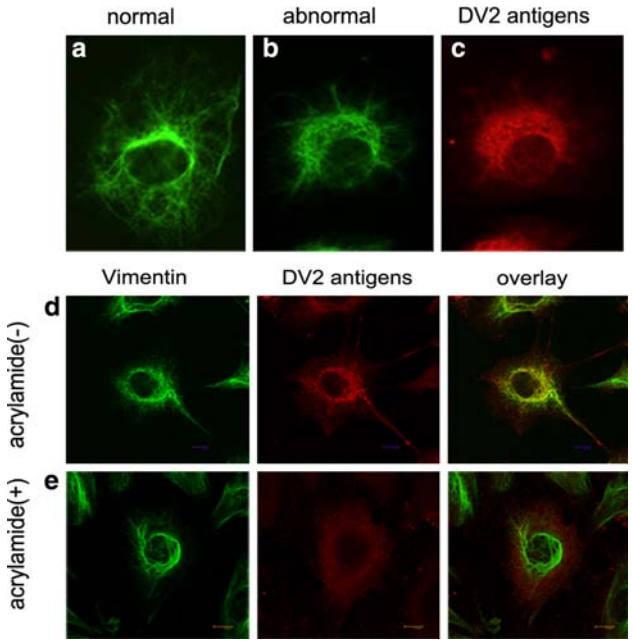

Fig. 3 Immunostaining images showing vimentin reorganization induced by DV2 infection and colocalization of DV2 antigens (*red*) with vimentin (*green*) in ECV304 cells at 24 h p.i. **a** Normal pattern of vimentin in mock-infected cells; **b**, **c** aberrant vimentin distribution and DV2 antigens in infected cells (magnification ×400). DV2 antigens often colocalized with vimentin in the untreated cells (**d**), while they were rarely seen to interact with vimentin in the acrylamide-treated cells (**e**). Bar represents 10 μm (**d**, **e**)

DV2 antigens with vimentin was rarely observed in acrylamide-treated cells. These results further suggest that vimentin is involved in DV2 infection.

In conclusion, our results indicate that vimentin is required for DV2 infection. MT may not be necessary for this process, but MT disruption may enhance DV2 release from infected cells. Further work is needed to demonstrate precisely what function these cytoskeletal proteins have during replication and with which DV2 proteins they interact.

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