Biochemical and Biophysical Research Communications **261,** 139–143 (1999)
Article ID bbrc.1999.0593, available online at http://www.idealibrary.com on IDEAL®

Induction of Manganese-Superoxide Dismutase in MRC-5 Cells Persistently Infected with an *Alphavirus,* Sindbis

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Received March 5, 1999

Sindbis virus (SV), a single-stranded positive-sense RNA virus, multiplies in a variety of cells and causes various outcomes of infection. As we described acute infection of SV induces stress response of small heat shock protein HSP27 and activation of mitogen-activated protein kinase (MAP) signaling pathway (Nakatsue, T., et al., *Biochem. Biophys. Res. Commun.* 253, 59–64, 1998). In contrast to lytic infection in Vero cells, MRC-5 cells, a human fetus lung cell line, resulted in persistent infection by SV. Here we investigated a cellular factor involved in persistent infection of MRC-5 cells infected with SV. Partial sequence analysis of a 25 kilodalton (kDa) protein, accumulated in large amounts in the cells, showed that manganese-superoxide dismutase (Mn-SOD) was induced during the infections. When Mn-SOD was overexpressed in Vero cells, 20% of the cells survived more than one month, in contrast with the death of 99% of the vehicle-transfected Vero cells at 48 h after infection with SV. These data strongly suggest that a cellular factor which regulates the oxidative pathway modulates the outcome of SV infection.

Sindbis virus (SV), family *Togaviridae,* genus *Alphavirus,* is small, enveloped, single-stranded positive-sense RNA virus. The virion structure, genome organization and replication mechanism have been extensively studied and well characterized in fibroblast cells, such as chick embryos, BHK-21 and Vero (1). SV infection in cultured cells presents an interesting and special situation: this virus has enormously wide host range that comprises both invertebrate and vertebrate hosts, and causes different types of infection such as acute (lytic), self-limited (incomplete or abortive) or persistent infection depending upon origins and physiological states of hosts (2, 3). For example, SV infection *in vitro* in cell lines and primary cultured neurons reveal two distinct outcomes of infection, lytic and persistent infection, respectively (4). In most epithelial cells in cultures, SV infection resulted in persistent or incomplete infection (5). Thus, various combinations of SV-host cells provide convenient model systems for investigating the viral and host determinants of the outcome of infection.

It has recently been reported that some cellular genes which are known to regulate the cell death pathway can modulate the outcome of SV infection in cultured cells. The cellular bax and bak genes, which are known to accelerate cell death, also accelerate virus-induced apoptosis (5, 6). We have shown that acute infection of SV induced stress responses such as phosphorylation and intracellular translocation of small heat shock protein HSP27, and activation of p38MAP kinase signaling pathway (7). This may contribute to the delayed onset of apoptosis in the host cells and facilitation of viral replication. In contrast, inhibition of apoptotic cell death such as bcl-2 gene product suppress virus-induced apoptosis, which can facilitate persistent viral infections *in vitro* (8) and *in vivo* (9).

To understand further relationship between SV and host, we studied a cellular factor which modulates viral replication and establishes persistent infection. Here we describe (i) another cellular factor, manganese-superoxide dismutase (Mn-SOD), is induced in MRC-5 cells persistently infected with SV, and (ii) Vero cells, which usually cause lytic infection with SV, overexpressed with Mn-SOD survived for long terms after infection of SV. It is generally accepted that cells are

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Abbreviations: SV, Sindbis virus; HSP, heat shock protein; kDa, kilodalton; MAPK, mitogen-activated protein kinase; Mn-SOD, manganese-superoxide dismutase; MOI, multiplicity of infection; PFU, plaque forming unit; Act. D, actinomycin D; PVDF, polyvinylidene di-fluoride; IF staining, immunofluorescent staining; PAGE, polyacrylamide gel electrophoresis; ROI, reactive oxygen intermediate.

0006-291X/99 $30.00

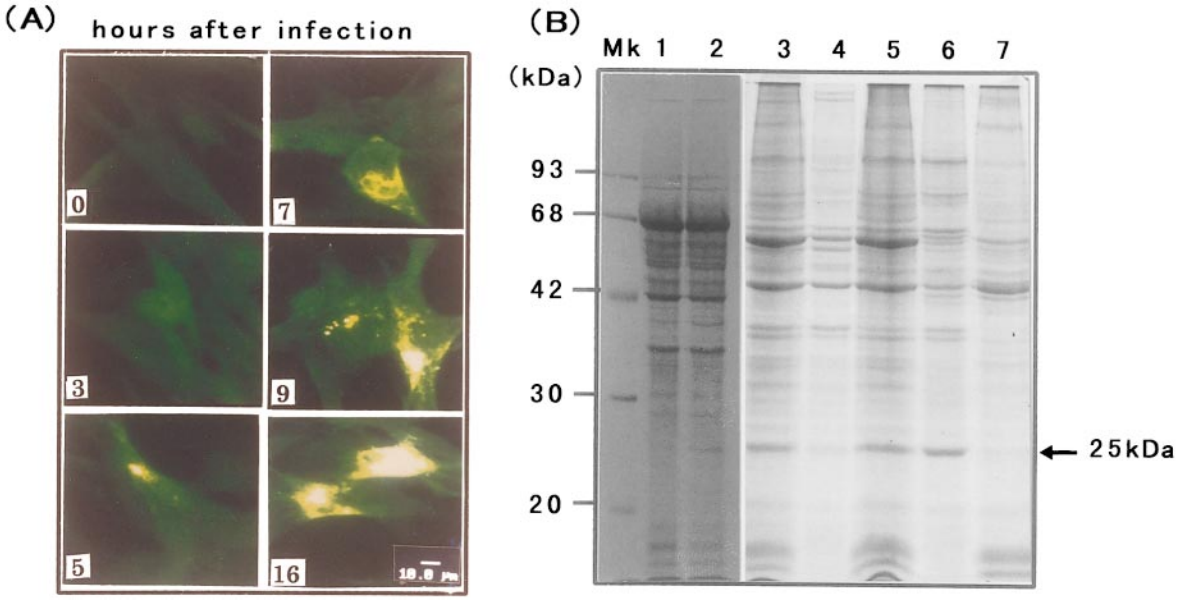

FIG. 1. SV growth and partial purification of 25 kDa protein in SV-infected MRC-5 cells. (A) MRC-5 cells infected with SV were fixed at the time indicated and stained with anti-SV ascites. (B) SDS-PAGE analysis of whole cell lyzates of uninfected (lane 1) and persistently infected cells obtained at 96 h postinfection (lane 2). Lanes 3 to 7: Whole homogenate of the persistently infected cells obtained at 10 days postinfection (3), supernatant of the homogenate (4) and pellets (5) at 10,000×g centrifugation, and soluble (6) and insoluble fraction (7) of (5) with NP40, respectively. Bar, 10 μm.

protected from free radical damage in part by an enzymatic antioxidant defense system, both copper, zinc- and Mn-SODs (10, 11). The result we obtained indicates that induction of Mn-SOD upon SV infection can be involved in the establishment of the persistent infection of MRC-5 cells through scavenging the free radicals produced during viral infection.

MATERIALS AND METHODS

Cells and virus. Cell lines, Vero (at passages 120), BHK-21 clone-13, NIH3T3/Swiss and MRC-5 cells were obtained from Dainippon Pharmaceutical Co., Ltd. and were grown as described (7). SV (strain AR339) was obtained from the National Institute of Infectious Diseases, Toyama, Tokyo, Japan. The propagation of viruses and determination of virus titers was described earlier (12). In experiments with actinomycin D (Act.D, Sigma), an appropriate concentration was included into the culture medium.

Protein analyses. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE), approximately 100 μg of total protein was loaded per slot (13). Gels were stained with Coomasie brilliant blue (CBB).

Cell fractionation. Cells grown in ϕ = 26 mm dishes were washed three times with PBS(−), scraped off by a rubber policeman into PBS(−), and collected by centrifugation. The cells were homogenized with 15 strokes of a Teflon-glass homogenizer in a lysis buffer (pH 7.2) containing 20 mM Tris, 0.1 M NaCl, and 1.5 mM $MgCl_2$. After centrifugation at 10,000×g for 20 min at 4°C, the pellets were extracted with lysis buffer containing 0.5% NP40, and centrifuged at 10,000×g for 20 min at 4°C. The supernatant was used for further analysis.

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GCGGGCGCGG -- --------- TAGCACCAG CACTAGCAGC ATG TTG  AGC CGG GCA GTG TGC   115
                                                Met  Leu  Ser  Arg  Ala  Val  Cys

GGC ACC AGC AGG CAG CTG GCT CCG GCT TTG GGG TAT CTG GGC TCC AGG CAG AAG CAC AGC   175
Gly  Thr  Ser  Srg  Gln  Leu  Ala  Pro  Ala  Leu  Gly  Tyr  Leu  Gly  Ser  Arg  Gln  Lys  His  Ser
                          -10                                                    1

AAG CAC CTG GAA CCT AGC CTC CCC GAC CTG CCC TAC GAC TAC  GGC GCC CGT GAA CCT CAC   235
Leu  Pro  Asp  Leu  Pro  TYr  Asp  Tyr  Gly  Ala  Leu  Glu  Pro  His   Ile  Asn  Ala  Gln  Leu  His

CAC GCG GCC TAC  GTG AAC AAC CTG - - - TGC AAA AAA TAA ACCACGATCG - - - - - AAA          1021
 His  Ser  Lys  His   His  Ala  Ala   Tyr ···  Cys  Lys  Lys  ***
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FIG. 2. Alignment of the determined NH_2-terminal amino acid sequences (underlined) of the 25 kDa protein with the amino acid sequence deduced from the DNA sequence (17).

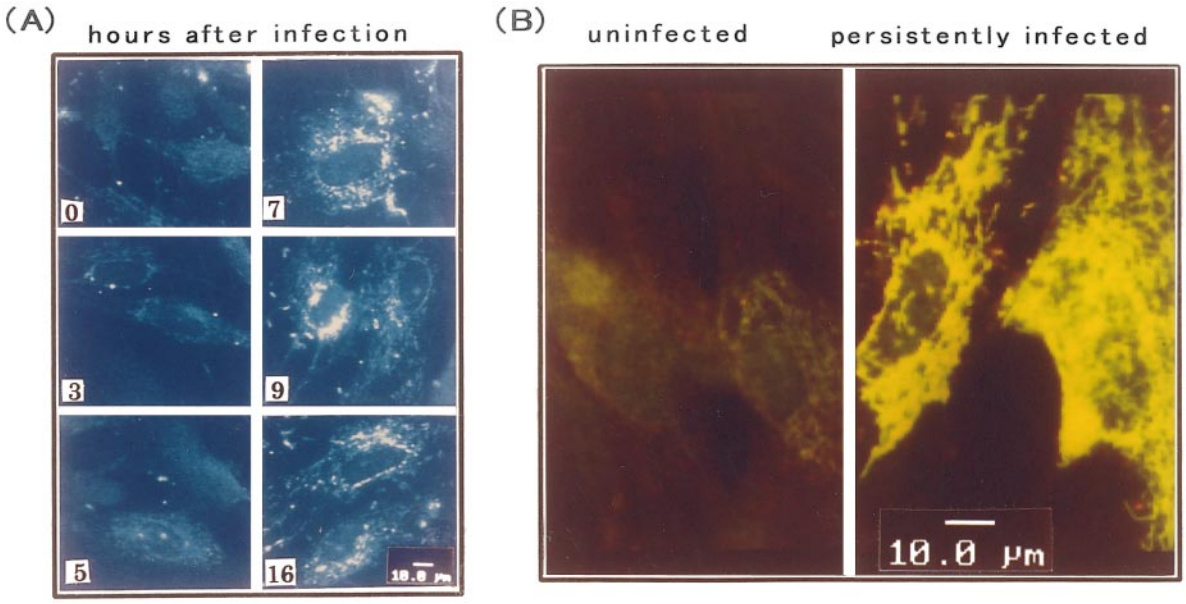

FIG. 3. Mn-SOD expression in MRC-5 cells during SV infection. Cells were stained with anti-Mn-SOD monoclonal antibody. (A) MRC-5 cells infected with SV were fixed at the time indicated, and stained with anti-Mn-SOD monoclonal antibody as in Fig. 1. (B) Uninfected and persistently infected MRC-5 cells (10 days postinfection) were stained with anti-Mn-SOD monoclonal antibody. Bar, 10 μm.

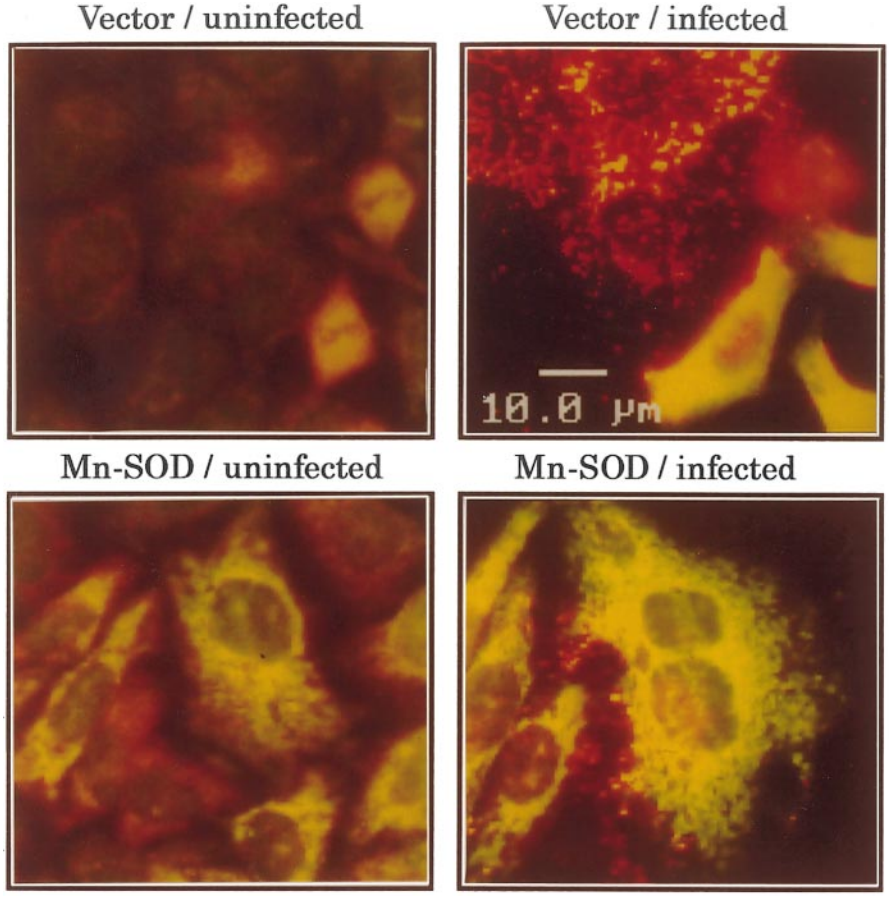

FIG. 4. Overexpression of Mn-SOD in Vero cells and SV growth in their cells. Vero cell clones stably transfected with human Mn-SOD gene were isolated and infected with SV. The cells were examined with IF staining to determine the levels of Mn-SOD expression and SV growth at 16 h postinfection as in Fig. 3. Virus antigens were stained in red (phycoerythrin) and Mn-SOD was stained in light green (FITC).

NH_2-terminal microsequence analysis. After the concentrated fraction of Mn-SOD was analyzed on SDS-PAGE, the protein was transferred to PVDF membrane following staining with ponceu S3. The stained band excised was analyzed by automated Edman degradation on an Applied Biosystems Protein Sequencer model 477A connected on line to a model 120A PTH Analyzer using an in-house generated gas phase program (14).

Construction of expression vectors. A human cDNA containing the entire Mn-SOD was kindly provided by Dr. N. Taniguchi (15). Expression vector was constructed using cytomegalovirus promoter (pTargeT, Promega), and named pTargeT-mSOD. The vector was introduced into Vero Cells, which expressed low levels of Mn-SOD, by conventional calcium phosphate precipitations (16). The pTargeT plasmid contains a neomycin-resistant gene, and transfected cells were cultured in the presence of 600 μg/ml of geneticin (Gibco-BRL) to select cells that incorporated the plasmids. After selection for 3 or 4 weeks, stable transfected clones were isolated.

Antisera and antibodies. Monoclonal antibodies for Mn-SOD (clone 31) was obtained from Calbiochem. This antibody was only suitable for IF staining. VR-1248F mouse ascitic fluid with immunization of SV was obtained from the American Type Culture Collections (ATCC).

Immunochemical staining of cellular proteins. Cells cultured on coverslips (ϕ = 18 mm) in 12-well dishes were washed with PBS(−), treated with 2% glutaraldehyde for 20 min, and permialized membrane with 1% Nonidet P40 (NP40) in PBS(−) (13). Fixed cells were incubated with an antibody diluted 1:100 in PBS(−) for 40 min at 37°C. After washing three times with PBS(−), the cells were further incubated with FITC or PE-conjugated secondary antibody, anti-mouse IgG, anti-sheep IgG, anti-goat IgG, or anti-rabbit IgG (Oregon Teknika Co. or Sigma chemicals). The secondary antibody was also diluted 1:100 with PBS(−). Coverslips were mounted in Crystal Mount (Biomedia Corp) and observed under a fluorescence microscopy (Zeiss microscope equipped with Meridian analytical apparatus).

RESULTS

SV growth in fibroblast cell lines and phenotype of infection. When fibroblast cell lines, including Vero, BHK-21 and NIH3T3/Swiss, were infected with SV at an MOI of 5 PFU, they produced maximum particles of 5×10^8–3×10^9 PFU per ml of the culture supernatant at 9 h postinfection and died in apoptosis within 48 h. Among 10 fibroblast cell lines were tested, MRC-5 cells, with one exception, were persistently infected with SV (Fig. 1A). The time course of the virus growth in MRC-5 cell infected with SV was similar to those of Vero cells, and reached the highest titers (10^8 PFU/ml, 1/10th of those in Vero cells) at 9 h postinfection, and 80% of the cells were infected at 48 h postinfection. Ninety-six hours after infection, 5–10% of cells survived and continue to grow, producing virus particles in lower amounts (10^4 PFU/ml). To examine the cellular defense mechanism of the infection we further cultivated the infected cells.

Induction of Mn-SOD in MRC-5 cells persistently infected with SV. Ten days after the initial infection the persistently infected MRC-5 cells, nearly confluent, were collected and protein compositions were analyzed by SDS-PAGE (Fig. 1B). A 25 kDa protein markedly increased (more than ten times as judged from the intensity of stained band) compared with the uninfected cells (Fig. 1B, lane 1). After fractionation of the cells (Fig. 1B, lane 3–7), the partially purified fraction containing concentrated 25 kDa protein (lane 6) was transferred to PVDF membrane, and the NH_2-terminal amino acid (AA) sequences of the protein were determined as described in Methods. Twenty amino terminal-AA residues were analyzed and aligned to the AA sequence deduced from the human Mn-SOD gene (17). The sequences obtained coincided with the NH_2-amino terminal AA (residues 25–45, underlined) in the mature Mn-SOD following the signal AA sequences (residues 1–24) in the precursor (Fig. 2). The estimated molecular weight calculated from AA compositions of the protein was 22,191. From these results we concluded that the 25 kDa protein is Mn-SOD.

Examination of Mn-SOD expression during SV infection by immunofluorescent (IF) staining revealed that the expression was gradually increased (5 fold at 9 h postinfection) during the course of the infection (Fig. 3A, 1–16), although uninfected MRC-5 also expressed reasonable amounts of Mn-SOD as basal levels (Fig. 3A, 0). In persistently infected cells, anti-Mn-SOD antibody stained cytoplasmic antigen in a dense mitocondrial pattern (Fig. 3B, 10 days post infection). To test whether Mn-SOD expression was involved in SV replication or not, Act. D was included in the culture. In the presence of the drug the virus growth was enhanced, as determined by 5-fold increase in IF staining, while expression of Mn-SOD was suppressed to uninfected cellular levels. In contrast with these, in the absence of the drug, expression of Mn-SOD was enhanced 10 fold at 16 h postinfection, and virus infected cells were reduced to 30% of the drug treated cells (data not shown).

Effects of the overexpression of the Mn-SOD on SV growth in Vero cells. Sixteen independent cell lines resistant to the antibiotic G418 were isolated from Vero cells treated with the plasmid pTargeT-mSOD. The cells were examined with IF staining to determine the levels of Mn-SOD expression in the parental control and transfected cells (Fig. 4). Seven transfectant clones constitutively expressed 5- to 10-fold higher levels of Mn-SOD compared with the parental cells (a typical example is shown in Fig. 4B). Cells expressing high levels of Mn-SOD and vehicle-transfected cells (Fig. 4A) were infected with SV. Virus growth and cell survival were determined by measuring the intracellular viral core antigens and the percentage of cells which were not stained with anti-SV ascites. Viral protein synthesis was delayed more than 3 h to reach the same level in control cells, and 30% of the cells were uninfected with strong staining with anti-Mn-SOD antibody at 16 h postinfection as to vehicle-transfected cells (data not shown). The maximum virus titer obtained at 16 h after infection was 5×10^7 PFU/ml, which was 1/10–1/20 of that in control cells.

The survived cells were alived at least one month, and continuously produced low amounts of viruses (10^3 PFU/ml), although cell growth rate was slow.

DISCUSSION

We studied the cellular factors that modulate viral growth and contribute as a non-specific cellular defense mechanism against viral infection. Recently, we have shown that specific stress responses, phosphorylation and intracellular translocation of HSP27 and activation of p38MAP kinase, are induced in lytic infection of SV-infected Vero cells (7). This response may contribute to the delayed onset of apoptosis in virus-infected cells. Here, we examined the cellular factor(s) involved in persistent infection of MRC-5 cells infected with SV. The cells that induced higher amounts of Mn-SOD upon SV infection were determined by analysis of a 25 kDa protein found in the cells. Furthermore, when Vero cells, which usually cause acute infection with SV, transfected with pTargeT-mSOD were infected with SV, 10% of the cells survived for more than one month in persistent infection. These results suggest that Mn-SOD may be responsible for the persistent infection of MRC-5 cells.

It is well known that Mn-SOD is induced in response to the acute inflammatory mediators, lipopolysaccharide (LPS), IL-1, TNF-α and other cytokines, and is one determinant of cellular resistance against reactive oxygen intermediates (ROI) through scavenging (18, 19, 20, 21). Upon virus infection, some cells respond to produce an acute inflammatory mediators such as interferon-γ, TNF-α and IL-6 (22, 23, 24). In MRC-5 cells, the suppression of Mn-SOD expression in the presence of Act. D during SV infection suggests that induction of Mn-SOD is a secondary effect of the infection. How Mn-SOD is induced is remains to be determined.

There has been some evidence that Mn-SOD might affect on viral replication. When influenza virus-infected mice or respiratory syncytial virus-infected cotton rats were administered SOD, it manifested significantly reduced inflammation, morbidity and mortality (25, 26). However, there is neither evidence that virus infection induced Mn-SOD might be an intrinsic cellular defense mechanism *in vitro* and in defend locally against viral infection *in vivo.*

In general, stress-induced proteins have protective functions against and/or repairs cellular damage (27). Taken together with our earlier result (7), this suggests that different stress responses are induced by SV infection in different cell lineages and physiological states. The outcomes of infection, lytic or persistent, might be determined depending on what stress responses are induced by SV infection.

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