Nip21 Gene Expression Reduces Coxsackievirus B3 Replication by Promoting Apoptotic Cell Death via a Mitochondria-Dependent Pathway

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Abstract—Our previous studies, using differential mRNA display, suggested that the mouse Nip21 gene may be involved in myocarditis development in the coxsackievirus B3 (CVB3)–infected mouse heart. Sequence comparison indicated that the mouse Nip21 gene shares high sequence homology to human Nip2. This human protein is known to interact with both the apoptosis inhibitor Bcl-2 and a homologous protein, the adenovirus E1B 19-kDa protein. Such interactions implicate Nip21 gene in cell death pathways. To study the function of this gene, we have cloned Nip21 from mouse hearts and established a Tet-On doxycycline-inducible HeLa cell line and a cardiomyocyte H9c2 cell line expressing Nip21 to characterize gene function in relation to apoptosis. We demonstrated that Nip21 expression could induce apoptosis via caspase-dependent mitochondria activation. To further determine the function of Nip21 in CVB3-induced apoptosis, the Tet-On/Nip21 HeLa cell line was induced by doxycycline followed by CVB3 infection. We found that activation of caspase-3 and cleavage of poly-(ADP-ribose) polymerase occurred 2 hours earlier than in vector-transfected control cells, suggesting that Nip21 expression enhances CVB3-induced apoptosis. We also demonstrated a significant decrease in HeLa cell and H9c2 cell viability. Particularly, as illustrated by viral plaque assay, CVB3 replication was dramatically reduced in Tet-On HeLa cells, due at least in part to the earlier killing of the host cells by Nip21 overexpression. (Circ Res. 2002;90:1251-1258.)

Key Words: Nip21 ■ apoptosis ■ myocarditis ■ coxsackievirus B3

Coxsackievirus B3 (CVB3)–induced myocarditis is a common heart disease in children and young people.1–3 Viral infection can cause rapid and severe cardiac dysfunction. Patients may recover or experience progressive disease, leading ultimately to end-stage dilated cardiomyopathy (DCM), which requires heart transplantation.4,5 The link between the myocarditis and the DCM is suggested based on the detection of viral genomic RNA in the heart tissue of patients with DCM6 and recently by in situ demonstration of viral capsid protein VP1 in myocardial tissues from DCM patients.7 These findings suggest that persistent viral infection plays an important role in the pathogenesis of DCM. However, the mechanisms by which CVB3 causes myocarditis and progression to DCM are poorly understood.

Although the immune or autoimmune response of hosts to viral infection is one of the known important processes in enteroviral myocarditis,8 viral direct cytotoxicity to cardiomyocytes is central to heart tissue injury.9 In cultured HeLa cells and cardiomyocytes, CVB3 infection induces a cytotoxic effect and cell death,10,11 and the production of defective CVB3 RNA in the heart of a transgenic mouse model leads to the development of a heart disease resembling DCM in humans.4 Enteroviral proteases have been reported to play an important role in viral pathogenesis. CVB3 protease 2A directly cleaves dystrophin, and impairs its functions in the cytoskeleton.12 Proteases 2A and 3C of poliovirus have been found to induce HeLa cell apoptosis.13,14 Our previous studies have also demonstrated that CVB3 infection can activate caspase-315 and induce cytochrome c release from mitochondria (unpublished data, 2002). In other reports, apoptotic cells were observed in inflammatory lesions as well as myocardial tissue outside inflamed areas, depending on the mouse strains and virus variants.16,17

Many factors influence host susceptibility to CVB3 infection and disease severity; however, the most important may be the host genetic background.17 The src family kinase Lck (p56lck) has been reported recently to be an essential host factor that influences replication and pathogenicity of CVB3 in vitro and in vivo, but p56lck is not a general regulator of enterovirus pathogenicity,18 implying a complicated interaction of CVB3 with host cells in the development of viral heart muscle disease.19 It is postulated that the pathogenesis of CVB3-induced heart diseases may be associated with differential expression of cellular genes controlling viral replica-
tion, infectivity, persistence and host gene transcription or translation as well as signal transduction in cardiomyocyte survival and programmed cell death.\textsuperscript{16,20–23} In an attempt to identify key genes in this disease, our previous studies by differential mRNA display have identified 5 differentially expressed genes in CVB3-infected mouse hearts.\textsuperscript{21} One of the downregulated genes is Nip21. However, the mechanism by which Nip21 participates in or counteracts viral infection is unknown. The Nip21 protein shares sequence homology with human BNip2 (also named Nip2), a molecule that can interact with adenovirus E1B-19K/Bcl-2 proteins involved in apoptosis pathways, implying a potential function for Nip21 in apoptosis. To test this hypothesis, in the present study, we established a Tet-On HeLa cell line and a cardiomyocyte H9c2 cell line expressing Nip21 and used these tools to determine the function of Nip21 in viral pathogenesis.

Materials and Methods

Cloning of the Nip21 Gene
cDNA encoding Nip21 was cloned by RT-PCR from total RNA isolated from the hearts of A/J(H-2\textsuperscript{a}) mice (Jackson Laboratory, Bar Harbor, Maine). The primer sequences used were 5'-CGTGAATTCGACATTGAGTCTAGG-3' and 5'-CGGTCTAGACCGGAACTACTTACT-3'. PCR fragments were cloned into both the Tet-On pTRE and pcDNA3 vectors at the EcoRI and XbaI sites. After sequence confirmation, the open reading frame of Nip21 was analyzed using the DNA Strider program. The predicted amino acid sequence was compared and aligned with other sequences in the GenBank using a BLAST alignment program (NCBI server).

Establishment of Tet-On/Nip21 HeLa Cell Line and H9c2/Nip21 Cardiomyocyte Cell Line

The inducible double-stable Tet-On/Nip21 cell line was established by cotransfection of Tet-On HeLa cells (Clontech, Palo Alto, Calif) with pTRE/Nip21 and pTK-Hyg plasmids using the LipofectAMINE (GIBCO) method and selection with hygromycin and G418. For generating Nip21-transfected cardiomyocytes, H9c2 cells (American Type Culture Collection, Manassas, Va) were differentiated in medium containing 1% of horse serum and 1 \textmu g/mL of retinoic acid for 4 days. The cells were then transfected with pcDNA3/Nip21 using the same method described for the HeLa cells. For both the Tet-On HeLa cells and H9c2 cells, the selected cells were further screened by a functional assay, i.e., detecting Nip21-induced cleavage of pro-caspase-3. The positive cell lines were finally confirmed by determination of Nip21 gene integration into cell chromosomal DNA by RT-PCR. The obtained positive cell lines were named Tet-On/Nip21 and H9c2/Nip21, respectively. Cells transfected with vector-alone were used as controls in later experiments.

Western Blot Analysis

Cell cultures were harvested at various time points after doxycycline (Dox)-induction or incubation. To determine the effect of Nip21 expression on CVB3-induced apoptosis, Dox-induced Tet-On/Nip21 HeLa cells were infected with CVB3 (Nancy strain) at a multiplicity of infection (MOI) of 10 before harvesting. Whole cell lysates were prepared in 1 mL of lysis buffer for detecting cleavage of pro-caspase-3 and caspase-3 substrates. To evaluate mitochondrial cytochrome c release, cytosolic extracts (S100) were prepared by centrifugation at 100 000g for 1 hour at 4°C. All the samples were fractionated and blotted onto a nitrocellulose membrane as described previously.\textsuperscript{15,18} The membrane was probed with primary antibody [mouse IgG to caspase-3 (Santa Cruz), poly(ADP)-ribosyl]polymerase (PARP), Bid, cytochrome c, and DNA fragmentation factor inhibitor (DFF-45/ICAD) (PharMingen), respectively) at room temperature for 2 hours. The signal was detected by the ECL method as per the manufacturer’s instructions (Amersham).

Caspase Activity Assay

The activity of caspase-3, -8, and -9 was measured using the ApoAlert kit (Clontech) following the manufacturer’s instructions. Cellular extracts were incubated with fluorescent substrates DEVD-AFC, IETD-A, and LEHD-AM, respectively, in the presence and absence of its specific inhibitor. Controls included the vector-alone–transfected cells and nontransfected HeLa cells treated with TNF-α/ cycloheximide (CHX) (positive). Samples were measured on a fluoromicroplate reader (TECAN). All experiments were performed in triplicate wells at least 3 times.

DNA Fragmentation Assay

Tet-On/Nip21 HeLa cells and control cells were grown in 6-well plates in the presence and absence of Dox at a final concentration of 1 \textmu g/mL. At 58 hours post induction (pi), the cells were harvested for genomic DNA isolation using a DNA Laddering kit (Clontech). DNA samples were analyzed by 1.6% agarose gel electrophoresis.

Cell Viability Assay

To determine the effect of Nip21 expression on cell viability, cell lines and their controls were cultured in the presence and absence of the general caspase inhibitor Z-VAD.fmk. Cell viability was determined using an MTS assay kit according to the manufacturer’s instructions (Promega). All cells were treated with MTS at different time points and submitted for absorbancy measurement at 490 nm. The cell viability of Dox-induced Tet-On/Nip21 cells and Nip21-transfected H9c2 cells was determined according to the absorbency corrected with background reading. Cell viability was expressed relative to vector-alone–transfected control cells.

Viral Plaque Assay

Tet-On/Nip21 and control HeLa cells were inoculated in 60-mm plates and induced with Dox. Three days later, cells were infected with CVB3 at a MOI of 10 and incubation was continued for 24 hours. The virus supernatants were obtained by freeze-thaw of the collected cells and centrifugation to eliminate cell debris. Viral plaque assay was performed using these supernatants following the method described previously.\textsuperscript{24} Supernatant from control HeLa cells was used as a control. The experiment was repeated 3 times.

Statistical Analysis

All values are presented as mean±SEM. Statistical significance was evaluated using the Student’s \textit{t} test for paired comparisons, with \textit{P}<0.05 considered statistically significant.

Results

Cloning of the Nip21 Gene From Mouse Heart

A 1011-bp long cDNA containing the entire Nip21 coding region was cloned by RT-PCR from the hearts of A/J (H-2\textsuperscript{b}) mice, an established CVB3-myocarditis model. DNA sequence determination indicated that this gene shares 99% sequence homology with \textit{Mus musculus} Nip21 (AF035207, unpublished). Further sequence comparisons with human genes revealed that it is 92% homologous to BNip2. The open reading frame of the Nip21 gene is 981 nucleotides long and encodes 326 amino acids. This deduced amino acid sequence shares 99% and 92% homology with \textit{Mus musculus} Nip21 and human BNip2, respectively. Figure 1A shows the sequence alignment of Nip21 and BNip2 and two common functional motifs, the putative Ca\textsuperscript{2+}-binding motif\textsuperscript{25} and the SHG tripeptide sequence, the latter of which was reported to participate in catalytic activity of cysteine protease.\textsuperscript{26} Amino acid comparisons also revealed that Nip21 is homologous (66% similarity in a 131 amino acid region) to the GTPase activating protein, RhoGAP (Figure 1B). This raises the
possibility that Nip21 may be involved in signal transduction pathways. The sequence has been deposited in GenBank (accession No. AF400107).

**Activation of Caspase-3 and Cleavage of Its Substrates**

To study the functional role of the Nip21 gene, a double-stable cell line overexpressing Nip21 in a tightly regulated manner was established. Using pro-caspase-3 cleavage as our screening parameter for Tet-On/Nip21 cell line, 11 clones were identified from 106 selected cell colonies. The positive clones were further confirmed by RT-PCR to detect the integration of the Nip21 gene into HeLa cell chromosomal DNA (data not shown). A positive cell line was used to perform functional analysis of Nip21 in CVB3-infected HeLa cells. Figure 2A shows Western blot analysis to detect the cleavage product of pro-caspase-3. This data demonstrates that Nip21 expression can induce caspase-3 activation and cleavage of PARP (Figure 2B), both events occurring at least 2 hours earlier than in control HeLa cells.

To determine whether the Nip21 gene itself could induce caspase-3 activation in the absence of CVB3 infection, the same Tet-On/Nip21 and control cell lines were used to perform these experiments. Western blot analysis demonstrated that Nip21 could induce pro-caspase-3 cleavage as evidenced by a significant increase of the cleavage product (17 kDa) at 24 and 72 hours pi (Figure 3A). Activation of caspase-3 was further verified by cleavage of its substrates, PARP and DFF-45/ICAD. Figure 3B shows Western blot analyses of the cleavage products of these substrates, which had an increase at 48 hours pi. However, Tet-On control cells did not show cleavage of either substrate.

**Caspase Activity, Bid Cleavage, and Cytochrome c Release From Mitochondria**

To determine whether Nip21 expression can induce caspase-3 activation in cardiomyocytes, a stably transfected H9c2 cell line was established. Caspase activity assays using an ApoAlert kit in H9c2/Nip21 cells as well as Tet-On/Nip21 cells were performed and demonstrated that Nip21 expression does induce caspase-3 activation in cardiomyocytes. Figure 4A shows that the H9c2/Nip21 cell extracts contained more than 4-fold caspase-3 activity than control cell extracts. Similarly, caspase-3 activity of Tet-On/Nip21 cells is 4.5-fold higher than that of control cells. To determine the activity of other caspases that could activate caspase-3, assays for caspase-8 and caspase-9 were also conducted on these cell lines. Figure 4B shows that caspase-8 activity in H9c2/Nip21 and Tet-On/Nip21 cells is 2- and 2.2-fold higher than its control, respectively. Caspase-8 activation was also assessed in Tet-On/Nip21 cells by the cleavage of its substrate Bid as shown in Figure 5A. Similarly, we demonstrated that caspase-9 activity in H9c2/Nip21 cells and Tet-On HeLa/Nip21 cells is 3.4- and 5.2-fold higher, respectively, as compared with its control (Figure 4C). To determine whether the activation of caspase-3 is induced directly by caspase-8 or caspase-9 or both, we further measured caspase-3 activity in the presence of these caspases.

analyses of the cleavage products of these substrates, which had an increase at 48 hours pi. However, Tet-On control cells did not show cleavage of either substrate.
of caspase-8 inhibitor (C8i) and caspase-9 inhibitor (C9i), respectively. The data in Figure 4A shows that C8i almost totally blocked caspase-3 activity in both cell lines. However, C9i only partially inhibited caspase-3 activity (decreased approximately 2.5-fold), indicating that caspase-3 activation is via caspase-8 pathway.

Because caspase-9 activation is dependent on mitochondrial cytochrome c release in most instances, we further tested whether Nip21 expression could induce cytochrome c release from mitochondria. Western blot using Tet-On/Nip21 cell extracts was performed and demonstrated that cytochrome c release from mitochondria occurred at 48 hours pi (Figure 5B). Because cytochrome c release from mitochondria may be via both caspase-8 pathway and overexpression of Nip21 protein, we further tested whether C8i can completely block the downstream caspase-9 activation induced by cytochrome c. Figure 4C demonstrated that caspase-9 activities in both Tet-On/Nip21 and H9c2/Nip21 cells were almost completely inhibited by C8i, indicating that caspase-9 activation is through cytochrome c release from mitochondria induced by cleaved Bid protein.

**Cell Viability**

Cell viability was measured using the MTS assay kit (Promega). Figure 6A indicates that at day 3 pi, approximately 60% cells are alive in Tet-On/Nip21 cells as compared with the control cells. However, in the culture to which Z-VAD.fmK was added, the viable cells were approximately 92% of the control HeLa cells. Similar results were obtained for H9c2 cell line, which demonstrated that after incubation for 4 days cell viability of H9c2/Nip21 was reduced to 58% of the control cells. However, the viability of Z-VAD.fmK–inhibited H9c2/Nip21 cells showed a notable increase to approximately 90% of the control cells (Figure 6B). This indicates that Nip21 expression induces cardiomyocyte apoptotic cell death.
Cell viability was also determined by observation of cell morphological changes and determination of chromosomal DNA fragmentation. The cell morphological changes were observed at different time points under a phase-contrast microscope. Figure 7A demonstrates that a number of alterations in cellular morphology are apparent on expression of Nip21. The loss of cell adherence and the appearance of cell shrinkage started 48 hours pi in Tet-On/Nip21 cells (Figure 7A-2). As a result, most of the cells were floating in the culture medium by 96 hours pi. For H9c2/Nip21 cells, the appearance of apoptotic morphology started 48 hours after incubation (Figure 7A-4). Analysis of cellular DNA shows that characteristic apoptotic DNA laddering appears mainly in Dox-induced Tet-On/Nip21 cells as compared with control cells (Figure 7B).

**Inhibition of Viral Replication**

The effect of Nip21 expression on CVB3 infectivity and replication was determined by a semiquantitative method to measure cell death on induction of Nip21 gene expression in CVB3-infected Tet-On/Nip21 cells and control cells. Figure 8A demonstrates that at day 4 after infection, CVB3 killed almost all of the control cells. However, in Tet-On/Nip21 cells, approximately 25% of the cells were still alive, indicating that CVB3 infectivity was significantly reduced by Nip21 gene expression.

The effect of Nip21 expression on CVB3 replication was also determined by plaque assays using virus supernatants obtained from CVB3-infected Tet-On/Nip21 cells and control cells. Figure 8B shows virus titer in plaque forming unit (pfu) per milliliter as obtained from triplicate experiments, in which the titer in Tet-On/Nip21 cells is 36% of that of the control cells, indicating that Nip21-expressing cells limit viral replication.

**Discussion**

In some virus-infected cells, apoptosis is initiated as a cellular defense mechanism to eliminate infected cells. A number of viruses encode proteins that suppress apoptosis resulting in efficient virus replication and pathogenesis. Human adenovirus E1B 19-kDa protein is one such protein that efficiently suppresses apoptosis manifested during viral infection. E1B 19-kDa protein is a functional homologue of the cellular antiapoptosis protein Bcl-2. However, how these proteins interact with other proteins to play a role in cell survival/death pathways remains to be elucidated.

In this study, we used A/J (H-2) mouse hearts, a well-established model of CVB3 myocarditis, to clone the Nip21 gene. Sequence determination and GenBank search suggested that Nip21 is a member of Bcl-2/adenovirus E1B 19-kDa-interacting protein family. Within this family, several human homologues have been reported, such as BNIp1 (previously Nip1), BNIp2, BNIp3, BNIp3h, BNIp3L, and Nix. Sequence comparisons indicate that mouse Nip21 is a homologue of human BNIp2, but is far less related to the other Nip proteins. Although BNIp2 has been suggested to be involved in cell death pathways, there is no direct evidence to support
this hypothesis. Mouse Nip21 protein sequence is 89% identical (92% similarity) to human BNip2, thus studies of this gene’s function will provide novel insight into our understanding of the mechanisms regarding how this group of proteins regulate cell survival or death.

Because Nip21 expression may be toxic to mammalian cells, we established a Tet-On/Nip21 expression system, in which Nip21 expression is under the control of an inducible promoter.29 By using this system, a series of experiments to detect cellular responses to Nip21-induced apoptosis were performed. Our data have demonstrated that Nip21 expression induces activation of caspase-3, cleavage of caspase-3 substrates, DNA fragmentation, and appearance of apoptotic cell death. Thus, Nip21 is a proapoptotic gene.

It is suggested that apoptotic pathways can be either caspase-dependent or caspase-independent.30 In caspase-mediated pathways, caspase-3 plays a pivotal executioner role in the apoptotic process. Two major pathways of caspase-3 activation have been described.31 The first pathway involves ligation of death receptors by their ligands, resulting in the recruitment of adapter proteins and pro–caspase-8 molecules to transactivate caspase-8.32 The activated caspase-8 may further trigger cleavage of Bid and in turn induce cytochrome c release from mitochondria.33 In the second pathway, cytochrome c release from the mitochondria is triggered by various cellular stresses. Transactivation of the complex pro-caspase-9 to active caspase-9 occurs after the release of cytochrome c, which binds to Apaf1, recruiting pro–caspase-9 molecules.34,35 In both pathways, once caspase-8 or -9 is activated, then it cleaves and activates caspase-3. In our functional evaluation of Nip21 expression, we showed that it induced caspase-3 activation in both HeLa cells and cardiomyocytes. We also showed the activation of caspase-8 and caspase-9, Bid cleavage, and cytochrome c release from mitochondria. We further demonstrated that C8i but not C9i could almost completely block caspase-3 activation. These data suggest that Nip21-induced caspase-3 activation is directly via both caspase-8 cleavage of pro–caspase-3 and the activation of caspase-9 by mitochondrial cytochrome c. Because cytochrome c release from mitochondria may be through both caspase-8–mediated Bid cleavage and overexpression of Nip21 protein, we further tested whether C8i can completely block the downstream caspase-9 activation. Our data demonstrated that caspase-9 activities in both Tet-On/Nip21 and H9c2/Nip21 cells were totally inhibited by C8i, suggesting that cytochrome c–dependent caspase-9 activation is triggered by cleavage of Bid protein by caspase-8.

Most notably, Nip21 expression in HeLa cells showed strong anti-CVB3 activity. We performed plaque assays on regular HeLa cell monolayers using supernatant harvested from CVB3-infected Tet-On/Nip21 cells and obtained significantly lower titers than that using the supernatant from vector-only–transfected control cells. When plaque assay was performed directly on Tet-On/Nip21 cell monolayers, it revealed that both the plaque number and size were smaller than those of control cells (data not shown). This is probably due, at least in part, to the fact that earlier apoptosis induced by Nip21 expression decreases the ability of host cells to foster virus replication and reinfection, therefore limiting the availability of released virus particles. This inhibitory effect of Nip21 on cell-to-cell viral infectivity was also observed in the semiquantitative measurement of cell death, in which approximately 25% Tet-On/Nip21 cells retained native morphology at day 4 after CVB3 infection. In contrast, almost all control cells are dead at this time point, indicating that Nip21 inhibition of viral replication and infectivity very likely occurs by promoting host cell death such that viruses are unable to produce sufficient progeny viruses for secondary infection.

It has been reported that human BNip2 shows a significant homology (19.7% overall identity on a 303 amino acid
target organ cells like cardiomyocytes, especially during the acute phase of infection. On the other hand, CVB3 infection can become persistent and enter a chronic phase.\textsuperscript{10,17} We do not know how Nip21 expression is regulated during the chronic phase or how Nip21 gene expression is regulated at the translational level. In addition, it is interesting to note that Nip21 shares high sequence homology with the GTPase-activating protein RhoGAP\textsuperscript{40} at a 131 amino acid region (47% identity, 66% similarity). The homology between these two proteins raises the possibility that certain signal transduction pathways may play a role in counteracting cell death--inducing stimuli. In this context, it has been reported that Bcl-2 associates with R-Ras,\textsuperscript{41} a member of the Ras superfamily. Nip21 may influence RhoGAP activity by interacting with Bcl-2, thus further affecting Rho and potentially other members of the Rho family.\textsuperscript{42} Interestingly, Nip21 also contains a putative Ca\textsuperscript{2+} binding motif (Figure 1A). Because intracellular Ca\textsuperscript{2+} appears to be an important mediator of apoptotic cell death, it is possible that the activity of Nip21 may be modulated by Ca\textsuperscript{2+} influx. It has been reported that CVB3 infection of cultured rat cardiomyocytes elevates the concentration of myocardial cytosolic free calcium and induces apoptosis.\textsuperscript{43,44} The mechanism has been related to CVB3 nonstructural protein 2B, which can modify plasma membrane permeability, thereby inducing increased levels of cytosolic free Ca\textsuperscript{2+}.\textsuperscript{45} Elevation of Ca\textsuperscript{2+} concentration in the cell may modulate the activity of Nip21 and in turn enhance the interaction between Nip21 and Bcl-2, thereby promoting cell death.

In conclusion, we have identified a proapoptotic gene Nip21 cloned from the heart of CVB3-infected mice. It appears that this gene induces apoptosis by activation of caspase-3. Further, the caspase-3 activation occurs via caspase-8--dependent mitochondria injury, cytochrome c release, and caspase-9 activation. Nip21 expression can inhibit CVB3 replication. However, whether this inhibitory action is directly or indirectly through an effect on viral transcription and/or translation needs to be studied further.

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