The role of IFN regulatory factor-3 in the cytotoxic activity of NS-9, a polyinosinic-polycytidylic acid/cationic liposome complex, against tumor cells

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Abstract

NS-9 is a complex of polyinosinic-polycytidylic acid and a novel cationic liposome, LIC-101. The complex has strong cytotoxic activity against tumor cells derived from epithelial or fibroblastic cells. We have investigated the mechanism of the cytotoxic activity of NS-9 using knockdown cells in which the expression of proteins of interest was inhibited by RNA interference. NS-9 showed strong cytotoxic activity against knockdown cells with reduced expression of double-stranded RNA-dependent protein kinase, RNase L, or IFN-α/β receptor, but showed no cytotoxic activity against IFN regulatory factor-3 (IRF3) knockdown cells. In IRF3-knockdown cells, NS-9 also did not induce either the DNA fragmentation or the rRNA degradation observed in negative control cells. We conclude that IRF3 plays a crucial role in the cytotoxic activity of NS-9 against tumor cells, whereas RNA-dependent protein kinase, RNase L, or type I IFNs are not important for its activity. [Mol Cancer Ther 2005;4(5):799–805]

Introduction

NS-9 is a complex of polyinosinic-polycytidylic acid [poly(I):poly(C)] and a novel cationic liposome, LIC-101 (1). The complex has strong cytotoxic activity against tumor cells of epithelial or fibroblastic origin. In a previous study (1), we showed that NS-9 induces apoptosis in tumor cells, including DNA fragmentation and rRNA degradation. Double-stranded RNA (dsRNA) such as poly(I):poly(C) is well known as a strong inducer of type I IFN (2). Both type I IFNs and dsRNA increase the expression of dsRNA-dependent protein kinase (PKR), which is activated upon binding to dsRNA (3–5). Activated PKR then inhibits protein synthesis through phosphorylation of a subunit of initiation factor eIF-2 (6–8). 2′,5′-Oligoadenylate synthetase is also activated upon binding to dsRNA, leading to the synthesis of 2′,5′-phosphodiester-linked oligoadenylates (2-5A) from ATP (9, 10). 2-5A activates 2-5A-dependent RNase (RNase L), leading to degradation of viral mRNA and cellular rRNA (10–14). Both the PKR pathway and the 2-5A system are part of the antiviral mechanism and have recently been found to be involved in apoptosis induced by type I IFN or dsRNA (15–27).

dsRNA is abundantly produced during viral infection, and poly(I):poly(C), a synthetic dsRNA, has been used to mimic virus infection in cells. Some transcription factors, such as nuclear factor κB and activator protein, are involved in the prompt induction of type I IFN in viral infection (28). IFN regulatory factor 3 (IRF3) also plays a significant role in the induction of type I IFNs (28). Although IRF3 is usually localized to the cytoplasm, upon phosphorylation, it is translocated into the nucleus, where it induces the transcription of various genes (28). Toll-like receptor 3 (TLR3) has been identified as a receptor for dsRNA, and it was found to be involved in IFN-β induction by dsRNA (29). Furthermore, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) has been identified as a protein that associates with TLR3, and it was found to be required for the induction of IFN-β by dsRNA (30, 31). TRIF is also required for the activation of IRF3 by dsRNA (30, 31), which indicates that the binding of dsRNA to TLR3 activates IRF3 through TRIF, leading to the induction of IFN-β.

Elbashir et al. (32) found that RNA interference occurred in mammalian cells when small interfering RNA (siRNA) was introduced into the cells. When a 21-bp dsRNA cognate with a specific mRNA is transfected into cells, the mRNA is degraded, leading to the inhibition of protein expression. dsRNA binds to the RNA-induced silencing complex, which breaks down RNA, upon which the dsRNA becomes single-stranded. The single-stranded RNA then binds to mRNA cognate with the dsRNA (33). Single-stranded RNA, which can form a dsRNA-like structure called short hairpin RNA (shRNA), also has an RNA interference effect (34). In the present study, a plasmid DNA expressing shRNA was used to knock down the mRNA corresponding to various proteins of interest. Because several proteins in the IFN/dsRNA pathway have been shown to be involved in antiviral activity or apoptosis induced by either viral infection or dsRNA, we investigated their involvement in the cytotoxic activity of NS-9 against tumor cells using knockdown cells in which...
the expression of these proteins was markedly reduced. We found that IRF3, but not other proteins in the pathway that were tested, plays a crucial role in NS-9-induced cell death.

Materials and Methods

Materials
NS-9 was prepared by Nippon Shinyaku Co., Ltd., Kyoto, Japan (1). Poly(I):poly(C) and Brij 35 were purchased from Sigma (St. Louis, MO), Zeocin, Trizol reagent, random primer, SuperScriptII and proteinase K were from Invitrogen (Carlsbad, CA), DMEM was from Nissui (Tokyo, Japan), pSilencer 2.0-U6 was from Ambion (Austin, TX), 1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan and proteinase inhibitor cocktail were from Nacalai Tesque (Kyoto, Japan), Immobilon-P membranes were from Millipore (Bedford, MA), and an ELISA kit for human IFN-β was from Fujirebio (Tokyo, Japan). Anti-caspase-3 antibody was from Cell Signaling Technology (Beverly, MA), anti-PKR (N-18), anti-actin (I-19), anti-IRF3 (FL-425), anti-IFNα/βRo (H-11), and peroxidase-linked anti-goat IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and peroxidase-linked anti-mouse or anti-rabbit IgG antibodies and the enhanced chemiluminescence plus Western blotting detection kit were from Amersham (Piscataway, NJ). Anti-RNase L antibody was provided by Dr. Robert Silverman of The Cleveland Clinic Foundation (Cleveland, OH), and all rights, title, and interest in the anti-RNase L antibody are owned by The Cleveland Clinic Foundation (Cleveland, OH). Anti-RNase L antibody was provided by Dr. Robert Silverman of The Cleveland Clinic Foundation (Cleveland, OH), and all rights, title, and interest in the anti-RNase L antibody are owned by The Cleveland Clinic Foundation (Cleveland, OH). Anti-RNase L antibody was provided by Dr. Robert Silverman of The Cleveland Clinic Foundation (Cleveland, OH), and all rights, title, and interest in the anti-RNase L antibody are owned by The Cleveland Clinic Foundation (Cleveland, OH).

Cell Culture
HeLa cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum and 0.06% (w/v) l-glutamine at 37°C in an atmosphere of 5% CO₂ in air.

Construction of shRNA Expression Vector
Modified pSilencer 2.0-U6 was constructed by inserting a BamHI/HindIII fragment derived from pUCBM21 into the BamHI/HindIII restriction site of pSilencer 2.0-U6. A shRNA expression vector, pS2U6-zeo, was constructed by inserting a Zeocin-resistance gene into the modified pSilencer 2.0-U6. Oligonucleotides coding for shRNAs were designed according to the manual for pSilencer 2.0-U6 and the oligonucleotides were cloned into the BamHI/HindIII restriction site of pS2U6-Zeo.

Generation of Knockdown Cells
Cells stably expressing shRNA were prepared as follows. shRNA expression vectors were transfected into HeLa cells with the transfection reagent LIC-101 (Nippon Shinyaku) and cells stably expressing shRNA established by selection in medium containing Zeocin (400 μg/mL). The expression of the mRNA and protein was tested in resistant colonies. The target sequences tested for knockdown of each gene are shown in Table 1. Knockdown cells were also prepared by treatment with siRNA/cationic liposome complex (siRNA/LIC-101). siRNA was constructed by mixing sense RNA with antisense RNA. siRNA/LIC-101 was added to cells at a concentration of 100 nmol/L (the concentration is given in terms of the base). The target sequences for the siRNA are also shown in Table 1.

Assessment of Cell Growth
Cells stably expressing shRNA were seeded onto a 96-well plate at a density of 3 × 10⁵ cells per well. Four hours later, NS-9 was added and the cells incubated for 3 days. 1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan was added to a concentration of 0.5 mg/mL, the cells were incubated for a further 4 hours, and the reaction was stopped by the addition of 10% (w/v) SDS containing 0.01 N HCl. The reaction mixtures were incubated for at least 10 hours at 37°C to solubilize the formazan product. Then the absorbance at 595 nm was measured and the percentage inhibition of cell growth calculated as described previously (ref. 1; the absorbance at 655 nm was used as the reference). For assessment of cells treated with siRNA/LIC-101, the cells were seeded onto a 96-well plate at a density of 2 to 4 × 10³ cells per well. After overnight culture, the cells were treated with 100 nmol/L siRNA/LIC-101 for 3 days. After washing with fresh medium, the cells were treated with NS-9 for 2 days at 37°C and the percentage inhibition of cell growth determined. The absorbance of control wells was almost the same for cells treated with GL3-siRNA/LIC-101 and cells treated with IRF3-siRNA/LIC-101.

Immunoblot Analysis
Cells were lysed with 50 mmol/L Tris-HCl (pH 7.5), containing 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1% (v/v) Nonidet P-40, 10% (v/v) glycerol and a protease inhibitor cocktail. Cell extracts were subjected to SDS-PAGE and the proteins were transferred to an Immobilon-P membrane. The membrane was first blocked with TBS containing 0.1% (w/v) Brij 35 and 5% (w/v) skim milk and then incubated with primary antibody. Antibodies conjugated with horseradish peroxidase were used to detect the primary antibodies. The proteins on the membrane were detected by enhanced chemiluminescence with an enhanced chemiluminescence plus Western blotting detection kit.

Reverse Transcription-PCR
Total RNA was extracted from cells with Trizol reagent and cDNA was synthesized from 2 μg of total RNA with random primer and superscript II according to the manufacturer’s instructions. The PCR primers for β-actin were 5'-CTTCTGACCAGCTAGTACC-3' (sense) and 5'-GTACACTTCCGACCTTCCG-3' (antisense) and for TLR3 5'-AGCCCTTCAACAGTGAGTC-3' (sense) and 5'-TCAACTGGGATCTCGTCAA-3' (antisense).

Microscopy of siRNA-Treated Cells
Cells were plated onto 60-mm culture dishes at a density of 2 × 10⁵ cells per dish and treated with 100 nmol/L GL3- or IRF3-siRNA/LIC-101 for 3 days. The cells were then washed with medium, treated with NS-9 at a concentration of 1 μg/mL for 6 or 10 hours, and photographed under an Olympus IX70 microscope.
**Table 1. Target sequences of proteins investigated**

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<th>Target gene</th>
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<td>RNase L</td>
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<td>BC000660</td>
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<td>GL3</td>
<td>U47296</td>
<td>6'&amp;AACACCUUGAAGAUAUCU9'16</td>
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**DNA Fragmentation**

Cells were lysed with 10 mmol/L Tris-HCl (pH 7.5), containing 200 mmol/L NaCl, 10 mmol/L EDTA, 0.2% (v/v) Triton X-100, and 0.1 μg/mL proteinase K. After incubation of the cells at 50°C for 10 hours, DNA was extracted with phenol and chloroform and precipitated with ethanol. The DNA was analyzed on a 1.8% (w/v) agarose gel and visualized under UV light.

*Analysis of rRNA*

Total RNA was extracted from cells with Trizol reagent and 5 μg samples were analyzed on a 1.8% (w/v) agarose gel containing 2.2 mol/L formaldehyde and visualized under UV light.

**Results**

**PKR, RNase L, and TLR3 Do Not Play Significant Roles in the Cytotoxic Activity of NS-9 against Tumor Cells**

We investigated whether PKR, RNase L, or TLR3 are involved in the cytotoxic activity of NS-9. To obtain the corresponding knockdown cells, we cloned HeLa cells that stably express shRNA for PKR, RNase L, or TLR3, leading to knockdown of the expression of the corresponding genes. We obtained some cloned cells in which the expression of PKR, RNase L, or TLR3 was undetectable or barely detectable (Fig. 1A–C). Despite the reduced expression of PKR, RNase L, or TLR3, NS-9 still had cytotoxic activity against them that was as strong as that against the parental HeLa cells (Fig. 1A–C). These results indicate that PKR, RNase L, and TLR3 did not play a crucial role in the cytotoxic activity of NS-9.

**Type I IFNs Are Not Involved in the Cytotoxic Activity of NS-9 against Tumor Cells**

NS-9 is a strong inducer of type I IFNs (1). We investigated whether NS-9-induced type I IFNs secreted into the medium are involved in the cytotoxic activity of NS-9. Because many type I IFNs may be induced by NS-9, we knocked down the expression of IFNAR1, a receptor for all type I IFNs in HeLa cells. As shown in Fig. 1D, even though IFNAR1 protein expression was undetectable in the cloned cells, NS-9 still had cytotoxic activity against them that was as strong as that against the parental HeLa cells. This result is consistent with our previous finding that anti-type I IFN antibody, which can neutralize the activity of type I IFN, does not reduce the cytotoxic activity of NS-9 (1). These results indicate that type I IFNs induced by NS-9 are not important for the cytotoxic activity of NS-9.

We also tested the cytotoxic activity of NS-9 against cells treated with siRNA/cationic lipidosome complex (siRNA/LIC-101). NS-9 had cytotoxic activity against cells treated with PKR- or IFNAR1-siRNA/LIC-101, even though the expression of the corresponding proteins was inhibited by treatment with siRNA/LIC-101 (supplemental data, Fig. 1A and B). These data are consistent with the results obtained by testing knockdown clones.

**IRF3 Is Required for the Cytotoxic Activity of NS-9 against Tumor Cells**

Attempts to construct IRF3-knockdown cells by transfection with shRNA expression vectors were not successful; the reason for this is currently under investigation. We therefore employed the alternative strategy of treating the cells with an siRNA/cationic lipidosome complex, siRNA/LIC-101. We treated HeLa cells with IRF3-siRNA/LIC-101 and confirmed that IRF3 protein expression was undetectable in the cells 3 days later (Fig. 2A). We measured the number of surviving cells 2 days after the addition of NS-9 to cells treated with either GL3-siRNA/LIC-101 (negative control) or IRF3-siRNA/LIC-101 (IRF3 knockdown). NS-9 had strong cytotoxic activity against negative-control cells, but no cytotoxic activity against IRF3-knockdown cells (Fig. 2B). This result indicates that IRF3 has a crucial function in the cytotoxic activity of NS-9. To confirm the result, we examined the morphology of negative-control and IRF3-knockdown cells. NS-9 did not induce any sign of cell death in IRF3-knockdown cells even 10 hours after its addition (Fig. 2C). In negative-control cells, however, NS-9 began to induce cell death 6 hours after its addition, and many dead cells were observed after 10 hours. We obtained the similar results in A549 lung carcinoma cells (supplemental data, Fig. 2A and B). We also tested the cytotoxic activity of NS-9 against cells treated with siRNA/cationic lipidosome complex (siRNA/LIC-101). NS-9 had cytotoxic activity against cells treated with PKR- or IFNAR1-siRNA/LIC-101, even though the expression of the corresponding proteins was inhibited by treatment with siRNA/LIC-101 (supplemental data, Fig. 1A and B). These data are consistent with the results obtained by testing knockdown clones.

**IRF3 Is Involved in NS-9-Induced DNA Fragmentation and rRNA Degradation**

We have previously shown that NS-9 induces DNA fragmentation in tumor cells, indicating apoptotic cell death (1). In the present study, we tested whether NS-9 induced DNA fragmentation in IRF3-knockdown cells. DNA fragmentation was induced by NS-9 in negative-control cells, but not in IRF3-knockdown cells (Fig. 3A), providing further evidence for the role of IRF3 in apoptosis.
apoptotic cell death induced by NS-9. Procaspase-3 is cleaved to enzymatically active caspase-3 by autoproteolysis, a step that is followed by DNA fragmentation (35). We also found that NS-9 activated procaspase-3 to caspase-3 in negative-control cells, but not in IRF3-knockdown cells, as judged by the fact that, after the addition of NS-9, the amounts of procaspase-3 were reduced only in negative-control cells (Fig. 3A). This result indicates that IRF3 is also involved in NS-9-induced caspase-3 activation. NS-9 also induced rRNA degradation in negative-control cells, but not in IRF3-knockdown cells (Fig. 3B), indicating that IRF3 is also involved in NS-9-induced rRNA degradation.

**The Induction of IFN-β by NS-9 Is Dependent on IRF3**

TLR3 has been reported to be involved in cytokine expression induced by dsRNA (29). In addition, IRF3 is an important transcription factor in dsRNA-induced IFN-β expression (28). We tested for the induction of IFN-β by...
NS-9 (300 ng/mL) in TLR3-knockdown or IRF3-knockdown cells. Poly(I):poly(C) did not induce IFN-β in either wild-type HeLa cells or negative-control cells (data not shown). NS-9 did induce IFN-β in TLR3-knockdown HeLa cells as well as in wild-type HeLa cells (Fig. 4A). In contrast, NS-9 did not induce IFN-β in IRF3-knockdown cells, although it did induce the production of IFN-β in negative-control cells (Fig. 4B). These results indicate that IRF3, but not TLR3, is involved in the induction of IFN-β by NS-9.

Discussion

We have investigated candidate proteins that might be expected to mediate the cytotoxic activity of NS-9 against tumor cells. We focused on PKR, RNase L, IFNAR1, TLR3, and IRF3 because these proteins are all involved in IFN/dsRNA pathways. To assess the importance of these candidate proteins for the cytotoxic activity of NS-9, we used cells in which the expression of the protein of interest was inhibited by RNA interference.

Although PKR, RNase L, and type I IFNs are known to be involved in apoptosis induced by viral infection or treatment with dsRNA (15–27, 36) we found that these proteins did not play a significant role in the cytotoxic activity of NS-9. We also found that TLR3 was not crucial for the cytotoxic activity of NS-9, even though TLR3 is known to be a receptor for dsRNA (29).

Because Heylbroeck et al. (37) showed that virus-induced apoptosis is inhibited by the expression of the Sendai virus C protein, which inhibits the activation of IRF3, we have been interested in the role played by IRF3 in dsRNA-induced apoptosis. We tested the cytotoxic activity of NS-9 against IRF3-knockdown cells and found that IRF3 played a crucial role in the cytotoxic activity of NS-9 (Fig. 2B and C). We also found that Adriamycin, another apoptosis-inducing drug, still had cytotoxic activity against IRF3-knockdown cells, which was as strong as that against the negative-control cells (supplemental data, Fig. 3), which shows that IRF3-knockdown cells are not resistant to killing in general. To check the validity of the IRF3 target sequence, we also tested the cytotoxic activity of NS-9 against IRF3-knockdown cells using another IRF3 siRNA that targets a different part of the sequence of IRF3. These alternative IRF3-knockdown cells were also insensitive to NS-9 (300 ng/mL) in TLR3-knockdown or IRF3-knockdown cells. Poly(I):poly(C) did not induce IFN-β in either wild-type HeLa cells or negative-control cells (data not shown). NS-9 did induce IFN-β in TLR3-knockdown HeLa cells as well as in wild-type HeLa cells (Fig. 4A). In contrast, NS-9 did not induce IFN-β in IRF3-knockdown cells, although it did induce the production of IFN-β in negative-control cells (Fig. 4B). These results indicate that IRF3, but not TLR3, is involved in the induction of IFN-β by NS-9.

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Therefore, the NS-9-resistant phenotype of IRF3-knockdown cells resulted from specific knockdown of IRF3 and not from nonspecific knockdown of another protein.

We have previously found that NS-9 induces DNA fragmentation and rRNA degradation in tumor cells, presumably representing the mechanism of NS-9-induced cell death (1). In the present study, we found that IRF3 is involved in the DNA fragmentation and caspase-3 activation induced by NS-9 as well. These results suggest that NS-9 initiates a novel apoptotic pathway by which caspase-3 is activated through IRF3, leading to DNA fragmentation. There are several reports of rRNA degradation in apoptotic cell death (38–41), and rRNA degradation is now also considered to be a characteristic feature of apoptosis. We also found that rRNA degradation induced by NS-9 is dependent on IRF3. Taken together, these findings suggest that IRF3 is a starting point for all pathways of cell death induced by NS-9 (Fig. 5). Our findings further suggest that there might be a novel mechanism by which either caspase-3 or some unknown RNase is activated through IRF3. Although IRF3 is generally thought of as a transcription factor that is involved in IFN-β gene induction, it is possible that IRF3 also has other functions related to cell growth or cell death.

Because TLR3 was identified as a receptor for dsRNA (29), cytokine induction by extracellular dsRNA has been extensively studied. Extracellular dsRNA activates IRF3 through binding to TLR3 in a TRIF-dependent manner to promote the induction of IFN-β. In the present study, we found that the induction of IFN-β by NS-9 was dependent on IRF3 but not on TLR3. These results indicate that intracellular dsRNA requires IRF3, but not TLR3, for the induction of IFN-β. Unknown dsRNA-binding molecules in the cytoplasm might therefore be involved in the activation of IRF3 (Fig. 5). Recently, retinoic acid-inducible gene I (RIG-I) was identified as an essential regulator for intracellular dsRNA-induced signaling, which results in the induction of IFN-β mRNA through the activation of IRF3 (42). RIG-I could be involved in the activation of IRF3 by NS-9.

In conclusion, we have shown that, whereas PKR, RNase L, or type I IFNs play no role in the cytotoxic

Figure 4. Involvement of IRF3, but not TLR3, in the induction of IFN-β by NS-9. A, TLR3-knockdown or wild-type HeLa cells were treated with 300 ng/mL NS-9 for 48 h. B, HeLa cells were treated with siRNA and NS-9 as for Fig. 2. After treatment of the cells with 300 ng/mL NS-9 for 48 h, the concentration of cytokine in the medium was measured by ELISA. The experiment was done twice, with similar results. One of these experiments is shown. Columns, the average of the values for two wells. Replicate values were within 5%.

Figure 5. Pathways of NS-9-induced cell death.
activity of NS-9 against tumor cells, IRF3 is required for the cytotoxic activity of NS-9. Furthermore, our work suggests the existence of a novel mechanism of cell death induced by intracellular dsRNA.

References


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