# Antisense RNA to Inducible Nitric Oxide Synthase Reduces Cytokine-Mediated Brain Endothelial Cell Death

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**ABSTRACT: We test whether inhibition of inducible nitric oxide synthase (iNOS) can exert a cytoprotective effect on cerebral endothelial cells upon stimulation by pro-inflammatory cytokines. Mouse brain endothelial cells were stably transfected to express an antisense RNA against iNOS driven by an endothelium-specific von Willebrand factor (vWF) promoter. Upon stimulation with tumor necrosis factor-α (TNF-α) plus interferon-γ (IFN-γ), antisense transfectants showed less iNOS enzymatic activity with less nitric oxide (NO) when compared to the sense control cells. Correspondingly, the antisense cells showed a reduced LDH release and less cytosolic content of oligonucleosomes. These findings establish a cell-specific antisense strategy and confirm the cytotoxic role of iNOS expression in cultured cerebral endothelial cells.**

**KEYWORDS: endothelial cells; interferon-γ; tumor necrosis factor-α; von Willebrand factor**

## INTRODUCTION

Nitric oxide (NO), synthesized through the enzymatic conversion of L-arginine and molecular oxygen to L-citrulline by nitric oxide synthases (NOS),[1] serves a number of physiological functions. Among the three NOS isoforms that have been identified, inducible NOS (iNOS)[2] was first cloned from mouse macrophages and, unlike the other two constitutive NOS isoforms, expresses only under stimulation—mostly by inflammatory signals such as cytokines and/or endotoxin— to massively produce NO. NO produced by iNOS in a number of disease models has been demonstrated to exert either cytotoxic[3] or cytoprotective[4] effects, depending on the cell types and experimental paradigms selected.

In the central nervous system, NO synthesized by iNOS may contribute to secondary tissue damage during the inflammatory response that occurs hours to days

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following the primary insults, including brain ischemia and trauma. A major target of such an inflammatory reaction is the cerebral endothelial cell, which plays a pivotal role in sustaining blood flow and nutrient supply for brain tissues including neurons and glia. In addition to the expression of iNOS in response to inflammatory signals, cerebral endothelial cells also constitutively express eNOS,[5] which could also release NO to function as a vasodilator and which may be beneficial in maintaining optimal cerebral blood flow.[6] Therefore, selective inhibition of iNOS sparing eNOS is an attractive approach to fully delineate the cytotoxic versus cytoprotective potential of NO. Herein we used a brain endothelial (Bend) cell line as a model system in which iNOS can be induced by synergistic exposure to pro-inflammatory cytokines.[7] Our findings suggest that reduction in NO synthesis by antisense RNA to the iNOS gene protects these cells against cytokine-mediated injuries.

## MATERIALS AND METHODS

### *Plasmid Construction*

Based on the published cDNA sequence of mouse macrophage iNOS (Genebank accession number M92649), two sets of primers were designed to amplify two 145 bp fragments from mouse genomic DNA via polymerase chain reaction (PCR), one complementary (forward primer: 5′-CGGGATCCCTCCGTGGAGTGAACAAGA-3′; reverse primer: 5′-GGGGTACCTTTACAGGGAGTTGAAGAC-3′) and one homologous (forward primer: 5′-CGGGATCCTTTACAGGGAGTTGAAGAC-3′; reverse primer: 5′-GGGGTACCCTCCGTGGAGTGAACAAGA-3′) to the sequence between 45 bp and 189 bp of the murine iNOS transcript. These two DNA fragments derived from PCR represent the antisense (complementary) and sense (homologous) sequences covering a portion of the 5′-untranslated region 66 bp upstream of ATG initiation codon. Recognition sequences of restriction endonucleases BamH I and Kpn I were incorporated into the 5′-ends of forward and reverse primers, respectively, in both primer pairs for efficient subcloning into expression vector. The conditions used to amplify iNOS antisense/sense fragments were as follows: the preincubation was at 94°C for 2 min, reaction mixtures were cycled 30 times at 50°C for 1 min (annealing), 72°C for 1 min (extension), and 94°C for 40 s (denaturation), with a final extension of 10 min at 72°C.

To allow the expression of iNOS antisense/sense constructs exclusively in endothelial cells, an endothelial cell–specific expression vector was constructed. This was achieved by replacing the cytomegalovirus (CMV) immediate-early promoter in pcDNA3.1 (Invitrogen, Carlsbad, CA) with the promoter of von Willebrand factor (vWF). A primer pair (forward primer: 5′-GAAGATCTTTAGCCGATCCATTCAACCC-3′; reverse primer: 5′-CTAGCTAGCATACCTTCCCCTGCAAATGAG-3′) was synthesized to generate a 750-bp vWF promoter out of mouse genomic DNA. Recognition sequences of restriction enzymes Bgl II and Nhe I were included in the 5′-ends of forward and reverse primers, respectively. PCR was performed using 1 μg mouse genomic DNA with reactions carried out for 30 cycles of 52°C annealing for 1 min, 72°C extension for 1 min, and 94°C denaturation for 40 s. This was followed by an extension of 10 min at 72°C. The amplified mouse vWF promot-

er was then subcloned into Bgl II/Nhe I sites of pcDNA3.1 vector previously digested with Bgl II/Nhe I to remove the CMV promoter.

### *Southern and Western Blotting*

Southern blotting was conducted according to standard protocols using the DIG Labeling/Detection Kit (Boehringer Mannheim, Indianapolis, IN). To detect the iNOS protein by Western blotting, primary antibody against mouse macrophage iNOS (Transduction Lab., Lexington, KY) was applied at 1:500 dilution with a working concentration of 0.5 μg/mL. The anti-rabbit IgG secondary antibody (NA934, Amersham Life Science Inc., Arlington Heights, IL) was used at 1:7500 dilution. The immunoreactive components were then detected with ECL Western blotting detection reagent (Amersham Life Science Inc.) and exposed to Kodak XRP-1 films. To detect eNOS, primary antibody against mouse eNOS (Transduction Lab.) was applied at 1:1000 dilution at a final concentration of 0.25 μg/mL. The anti-rabbit IgG secondary antibody (NA934, Amersham Life Science Inc.) was used at 1:5000 dilution.

### *Statistical Analysis*

Results are expressed as means ± SD. Statistical analysis was performed using Student's unpaired *t*-test between two experimental groups (e.g., sense vs. antisense). Multiple groups were analyzed by one-way analysis of variance followed by a post-hoc Bonferroni *t*-test. A $P$ value of less than 0.05 was considered significant.

## RESULTS AND DISCUSSION

Exposure of Bend cells to a combination of TNF-α and IFN-γ resulted in a marked increase in the accumulation of nitrite in the medium as compared to those cells treated with serum-free medium only (FIG. 1A). Neither cytokine alone was capable of inducing NO production in Bend cells (data not shown). Additional NOS enzyme activity assay indicates that the accumulation of nitrite in the transfectants following cytokine induction was most likely due to increased expression of iNOS proteins, but not constitutive eNOS (data not shown). Exposure of Bend cells to cytokines led to cell death that was apparent after 24 h based on the LDH assay (FIG. 1B) and persisted until at least 48 h by MTT reduction assay (FIG. 1C).

To establish the causal relationship between TNF-α/IFN-γ–induced iNOS expression and its cytotoxicity toward brain endothelial cells, an antisense approach was adopted. A recombinant plasmid was constructed to drive the expression of an iNOS antisense RNA by vWF promoter to constitutively inhibit iNOS translation. In pilot experiments, transient transfection with a reporter gene construct (LacZ) driven by this vWF promoter has confirmed that this endothelial-specific expression vector was indeed functional in Bend cells (data not shown). After stable transfection with iNOS antisense construct, one transfectant, designated as "antisense," was isolated for further characterization. In addition, another colony of Bend cells transfected with corresponding iNOS sense construct was also isolated (designated as "sense") to serve as a negative control. Genomic DNA isolated from both antisense and sense transfectants, as well as that from parental Bend cells, were subjected to Southern

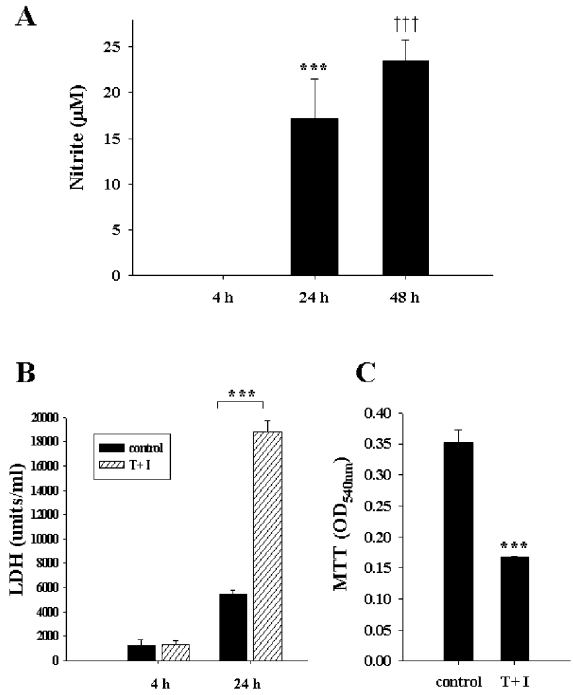


**FIGURE 1.** Increase in nitrite content and cell death induced by TNF-α/IFN-γ in Bend cells. (**A**) The nitrite contents in the medium from parental Bend cells following TNF-α (20 ng/mL)/IFN-γ (400 U/mL) treatment for 4, 24, and 48 h are shown. ***$P < 0.001$ compared to 4 h; †††, $P < 0.001$ compared to 4 h. (**B**) The extent of cell death based on LDH released in the parental Bend cells with (T + I) and without (control) TNF-α/IFN-γ. ***$P < 0.001$. (**C**) The extent of cell death based on the MTT assay with (T + I) or without (control) TNF-α/IFN-γ treatment for 48 h. Higher $OD_{540nm}$ absorbency on the *ordinate* indicates higher survival rates. ***$P < 0.001$. Mean ± SD in triplicate. Data shown are representative of three separate experiments with similar results.

blotting, using a 2.2-kb fragment containing the ampicillin resistance ($Amp^r$) gene as a probe, to ensure the stable integration of transgene sequences into the genome of transfectants. The left panel of FIGURE 2A shows the Hinc II and Sty I digestion patterns of genomic DNA isolated from both transfectants and parental Bend cells. The same gel was then subjected to Southern hybridization (right panel, FIG. 2A). A DIG-labeled probe only recognized the bands in genomic DNA isolated from antisense and sense cells, but not that from parental Bend. Western blotting demonstrated that, under identical experimental conditions with equal amounts of proteins (20 μg) loaded in each lane, antisense transfectants expressed substantially fewer iNOS proteins compared to sense controls (FIG. 2B). Densitometry scanning of the blot revealed approximately 60 to 70% inhibition of iNOS protein expression in antisense cells compared to sense or parental Bend cells. The blockade of iNOS expression in antisense cells is significant yet incomplete because an increase in iNOS

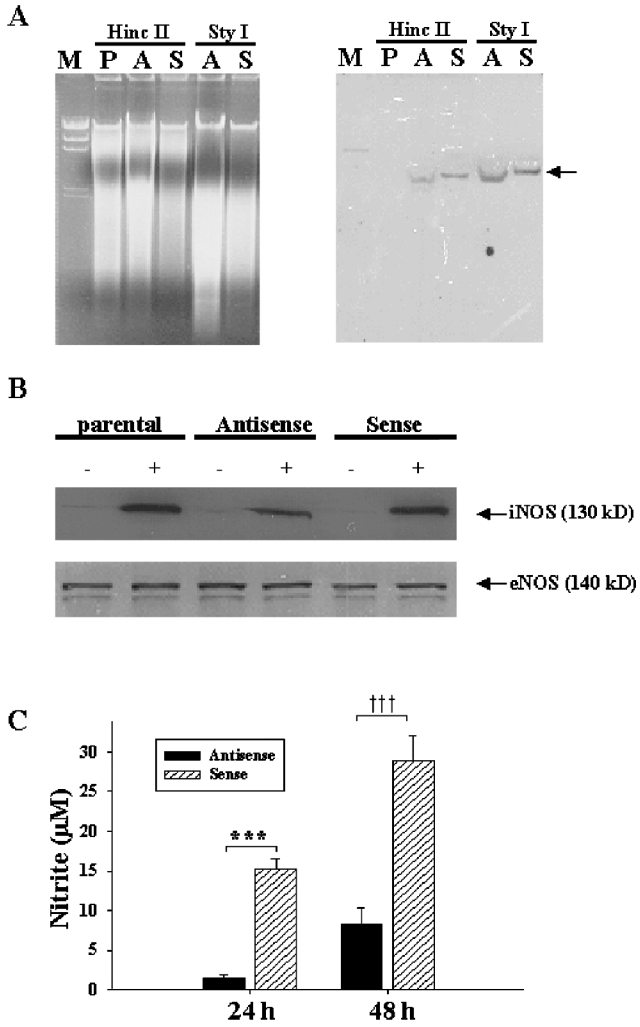


**FIGURE 2.** Characterizations of iNOS antisense and sense transfectants. (**A**) (*Left panel)* Agarose gel electrophoresis of genomic DNA digested with Hinc II and Sty I. *(Right panel)* The same gel blotted to a nylon membrane and probed with a DIG-labeled $Amp^r$ sequence. M, molecular weight markers of λ DNA digested with Hind III; P, genomic DNA of parental Bend cells; A, genomic DNA of antisense transfectants; S, genomic DNA of sense transfectants. The *arrow* indicates the hybridization signal. (**B**) Western blots of iNOS and eNOS with (+) or without (–) TNF-α (20 ng/mL)/IFN-γ (400 U/mL) treatment for 24 h. (**C**) The nitrite content in the medium from transfectants treated with TNF-α/IFN-γ for 24 and 48 h. ***$P < 0.001$; †††, $P < 0.001$. Results shown in (**C**) are representative of three separate experiments. Mean ± SD in triplicate.

expression could still be detected after TNF-α/IFN-γ treatment. As shown in the lower panel of FIGURE 2B, the eNOS level, however, did not show significant changes either in antisense or sense transfectants exposed to cytokines. Moreover, the eNOS levels between uninduced antisense and sense transfectants appear similar, indicative of a specific inhibitory effect of the antisense RNA against endogenous iNOS gene without affecting eNOS expression. Results in FIGURE 2C demonstrate that nitric oxide production, as reflected by the accumulation of nitrite contents in the medium, was partially suppressed in the stable transfectants of iNOS antisense construct upon cytokine induction, as compared to those transfected with sense iNOS construct. At the end of 24-h treatment with TNF-α/IFN-γ antisense cells produced significantly less nitrite than sense controls (antisense: $1.49 \pm 0.37$ μM; sense: $15.24 \pm 1.32$ μM, $P < 0.001$). After 48 h, culture media from antisense cells contained $8.31 \pm 1.99$ μM nitrite whereas those from sense contained $28.95 \pm 3.07$ μM ($P < 0.001$). In addition to the Griess reaction, NOS activity assay was performed with cytosolic extracts from transfectants following exposure to cytokines for 24 h. We found antisense cells did contain less calcium-independent NOS activity, presumably derived from iNOS, than that from sense controls (data not shown). In contrast, neither antisense nor sense transfectants showed significant changes in calcium-dependent NOS activities (presumably from eNOS) upon stimulation with cytokines (data not shown). Altogether, results in FIGURE 2 demonstrate that stable integration and overexpression of this 145-bp antisense gene is capable of suppressing, at least in part, iNOS enzymatic activity and NO synthesis upon cytokine induction.

We examined whether antisense suppression of iNOS expression may confer cytoprotection to Bend cells challenged with cytokines. A quantitative indicator of cell death is the oligonucleosomes released into cytosol of apoptotic cells.[8] Therefore, a sandwich ELISA kit was employed to quantitatively measure the contents of cytosolic oligonucleosomes in antisense and sense transfectants following cytokine exposure. As shown in FIGURE 3A, the relative oligonucleosome content in sense transfectants is much higher than those in the antisense cell line, although both cell lines had comparable background levels of cell death without cytokine induction ($1.09 \pm 0.17$ in antisense vs. $0.98 \pm 0.20$ in sense, $P = 0.4331$). This observation implies that excessive nitric oxide produced from iNOS as a result of cytokine induction may be detrimental to Bend cells. Antisense cells also demonstrated a relatively higher survival rate than did sense cells following cytokine stimulation based on LDH (FIG. 3B) and MTT (FIG. 3C) assays. We chose PI-staining of dead cells as a morphological approach to further analyze cell death (FIG. 3D). Under fluorescent microscope, very few cells were stained with PI in the antisense (upper left) and sense (lower left) transfectants without cytokines. With cytokine treatment, antisense cells (upper right) maintained a much higher cell viability as compared to sense cells (lower right) that show intense nuclear PI-staining, indicative of extensive cell loss.

Despite the constitutive expression of antisense RNA against iNOS, the antisense transfectants still expressed a small pool of iNOS proteins upon exposure to TNF-α/IFN-γ (FIG. 2B). However, TNF-α/IFN-γ did not cause an additional increase in cellular NO contents based on measurement of medium nitrite accumulation (FIG. 2C). The discrepancy between the extent of iNOS protein expression and medium nitrite accumulation remains unknown. Notably, expression of iNOS proteins may not necessarily cause massive NO production with resultant nitrite accumulation. Changes

in intracellular NO contents also depend on other factors. These include substrate availability, enzymatic activities of iNOS proteins, expression levels and/or enzymatic activities of other constitutive NOS isoforms. Interestingly, iNOS activity is subject to positive feedback regulation by NO. For example, pretreatment of rat hepatocytes with *S*-nitroso-*N*-acetyl penicillamine (SNAP), an NO donor, enhanced

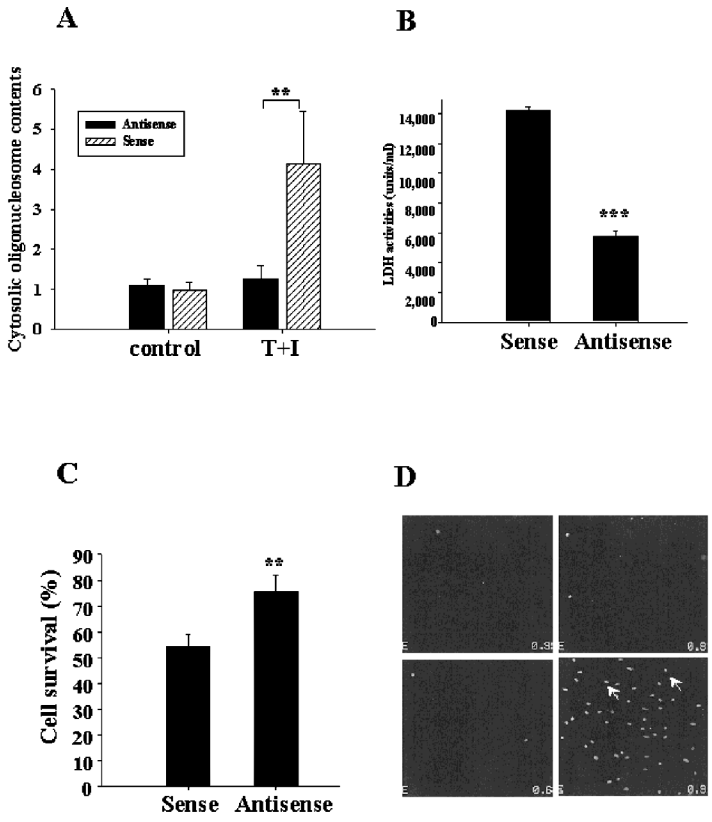


**FIGURE 3.** The extent of cell death in antisense and sense transfectants following cytokine exposure. (**A**) The DNA fragmentation ELISA assay with (T + I) or without (control) TNF-α (20 ng/mL)/IFN-γ (400 U/mL) treatment for 24 h. The results are expressed as relative cytosolic oligonucleosome contents in arbitrary units with serially diluted apoptotic HL60 cell lysate as reference standards. A higher value on the ordinate reflects a greater extent of cell death. $^{**}P < 0.01$. (**B**) The LDH assay following cytokine treatment for 24 h. $^{***}P < 0.001$. (**C**) The MTT assay following cytokine treatment for 48 h. $^{**}P < 0.01$. Mean ± SD in triplicate. Data shown in **A**, **B**, and **C** are representatives of at least three separate experiments with similar results. (**D**) *(Left panels)* The fluorescent images of the antisense (*upper*) and sense (*lower*) transfectants without cytokine treatment. (*Right panels)* The images of antisense (*upper*) and sense (*lower*) transfectants with cytokine treatment for 24 h. *Arrows* indicate the dead or dying cells with propidium iodide stain.

NO production by promoting iNOS dimerization without affecting expression level of iNOS mRNA or protein.[9] Partial suppression of iNOS expression may therefore abolish this potentiation effect of NO, rendering the remaining small portion of iNOS proteins incapable of massive NO production. This may in part explain the observation that, despite the presence of remaining iNOS proteins as detected by Western blotting (FIG. 2B), antisense RNA against iNOS effectively conferred cytoprotection to Bend cells upon exposure to TNF-α plus IFN-γ, based on LDH release assay (FIG. 3B). Another possibility is that the small amount of iNOS that remained in antisense transfectants, as well as the endogenous eNOS, contributed to the basal levels of NO that may be beneficial against the insults. NO at physiological concentrations is known to protect the human monocytic U937 cells against TNF-α–induced apoptosis by reducing the generation of ceramide.[10] Antisense suppression of iNOS with resultant reduction of excessive NO formation may therefore modulate cellular NO content to a lower level that was beneficial to Bend cells, causing cytoprotective effects against inflammatory cytokines such as TNF-α and IFN-γ.

Previously, we have successfully suppressed iNOS mRNA and protein expression induced by TNF-α/IFN-γ in Bend cells using a NF-κB trap.[7] This strategy entails exogenous introduction of a hairpin oligonucleotide decoy with the consensus sequence for NF-κB binding site. However, NF-κB is a potent transactivator for the expression of a number of other genes besides iNOS; downregulation of NF-κB thus appears to be a nonspecific—hence, a less ideal—approach to elucidate the cytoprotective or cytotoxic effect of iNOS.[11] In order to circumvent these problems, we transfected the murine brain endothelial cells with recombinant constructs that constitutively express the antisense RNA to iNOS gene driven by the promoter of endothelium-specific vWF. This 750-bp vWF promoter has previously been demonstrated to target the expression of LacZ to a subpopulation of endothelial cells in yolk sac and adult brain *in vivo*.[12] Therefore, this promoter may be especially valuable in gene therapy for treating cerebral disorders that involve endothelial cells. This vWF promoter has also been engineered to confer tissue-specific expression of a suicidal gene in human umbilical vascular endothelial cells as an anti-angiogenesis approach for cancer treatment.[13] Another attractive approach recently available for gene knockdown is the small interference RNA (siRNA). The endothelium-specific promoter may also be applied to drive the expression of siRNA *in vivo* for clinical use in the near future. Overall, results reported here and by others lay the foundation for future *in vivo* studies, using alternative gene delivery techniques such as retroviral or adenoviral vector, to suppress the iNOS activity in a cell-specific manner.

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## REFERENCES


1. PALMER, R.M. *et al.* 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature **327:** 524–526.
2. XIE, Q.W. *et al.* 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science **256:** 225–228.
3. HSU, C.Y. *et al.* 1997. Enhancement of apoptosis in cerebral endothelial cells by selected inflammatory signals. Ann. N.Y. Acad. Sci. **843:** 148–153.
4. RAUHALA, P. *et al.* 1998. Neuroprotection by S-nitrosoglutathione of brain dopamine neurons from oxidative stress. FASEB J. **12:** 165–173.
5. TOMIMOTO, H. *et al.* 1994. Distribution of nitric oxide synthase in the human cerebral blood vessels and brain tissues. J. Cereb. Blood Flow Metab. **14:** 930–938.
6. HUANG, P.L. *et al.* 1995. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. Nature **377:** 239–242.
7. XU, J. *et al.* 1997. Regulation of cytokine-induced iNOS expression by a hairpin oligonucleotide in murine cerebral endothelial cells. Biochem. Biophys. Res. Commun. **235:** 394–397.
8. XU, J. *et al.* 1998. Involvement of de novo ceramide biosynthesis in tumor necrosis factor-α/cycloheximide-induced cerebral endothelial cell death. J. Biol. Chem. **273:** 16521–16526.
9. PARK, J.H. *et al.* 2002. Nitric oxide (NO) pretreatment increases cytokine-induced NO production in cultured rat hepatocytes by suppressing GTP cyclohydrolase I feedback inhibitory protein level and promoting inducible NO synthase dimerization. J. Biol. Chem. **277:** 47073–47079.
10. DE NADAI, C. *et al.* 2000. Nitric oxide inhibits tumor necrosis factor-alpha-induced apoptosis by reducing the generation of ceramide. Proc. Natl. Acad. Sci. USA **97:** 5480–5485.
11. WRIGHTON, C.J. *et al.* 1996. Inhibition of endothelial cell activation by adenovirus-mediated expression of I kappa B alpha, an inhibitor of the transcription factor NF-kappa B. J. Exp. Med. **183:** 1013–1322.
12. AIRD, W.C. *et al.* 1995. Human von Willebrand factor gene sequences target expression to a subpopulation of endothelial cells in transgenic mice. Proc. Natl. Acad. Sci. USA **92:** 4567–4571.
13. OZAKI, K. *et al.* 1996. Use of von Willebrand factor promoter to transduce suicidal gene to human endothelial cells, HUVEC. Hum. Gene Ther. **7:** 1483–1490.