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¹안호준, ¹김택상, ¹강주석, ¹류현열, ²정헌택

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Regulatory Mechanisms for the Expression of Inducible Nitric Oxide Synthase (iNOS) Gene in Rat Vascular Smooth Muscle Cells

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Running title : Regulatory Mechanisms for the Expression of iNOS Gene in VSMC

Abstract

Background: The expression of inducible nitric oxide synthase (iNOS) in the vascular smooth muscle cells (VSMCs) is dependent on the multiple regulatory mechanisms of which are largely unknown. In this study, we focused on the role of IFN- γ in IL-1 β -mediated expression of the VSMCs iNOS gene.

Materials and Methods: VSMCs were isolated from rat thoracic aorta by enzymatic dissociation. Phosphorothioate-modified ODNs were designed to block initiation of target mRNA translation. Nitrite concentrations were measured by mixing 100 μ L of culture medium with 100 μ L of Griess reagent. The biological activity of IFN- γ was demonstrated by testing the effect of supernatants collected from the IL-1 β -treated VSMCs on the production of NO in RAW264.7 cells stimulated with LPS. Supernatant levels of IFN- γ were measured by Endogen Rat Interferon gamma enzyme-linked immunosorbent assay kit. Total RNA was prepared from cultured VSMCs using RNazol (Tel-Test) 12 hours after IL-1 β treatment. RNA (1 μ g) from each sample was reverse-transcribed with Oligo-dT as the first-strand cDNA primer and Maloney murine leukemia virus reverse transcriptase. Western blotting and electrophoretic mobility shift assay were performed.

Results: In a system of rat VSMCs in primary culture, we found that IL-1 β induced the productions of both NO and IFN- γ . IFN- γ protein and mRNA were expressed in the IL-1 β -stimulated VSMCs. Anti-IFN- γ antibody or antisense IFN- γ oligodeoxynucleotide (ODN) markedly inhibited NO production and iNOS protein expression as well as IFN- γ synthesis in the IL-1 β -stimulated cells. Furthermore, antisense IFN- γ ODN abolished the IL-1 β -induced activation of interferon regulatory factor-1 (IRF-1) without affecting NF- κ B activity as determined by electrophoretic mobility shift assay. The repletion of IFN- γ to the system restored NO production and IRF-1 activity.

Conclusion: These results suggest that the expression of iNOS gene in the IL-1 β -stimulated VSMCs is dependent on the endogenous IFN- γ synthesis and regulated through the activations of both IRF-1 and NF- κ B.

Key words : VSMCs, iNOS, IL-1, IFN- γ , IRF-1

Introduction

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The expression of inducible nitric oxide synthase (iNOS) gene generally requires exposure of cells to immune or inflammatory stimuli.¹⁾ This inductive phenomenon was

first demonstrated with mouse peritoneal macrophages treated with lipopolysaccharide (LPS), in which the expression of iNOS gene was enhanced synergistically by the addition of IFN- γ .²⁾ Synergistic enhancement of iNOS gene expression has been demonstrated in other cell types such as mesenchymal and epithelial cells exposed to two or more stimuli.³⁾ These studies have indicated that IFN- γ serves as primary activator in the expression of iNOS gene.⁴⁾ In vascular smooth muscle cells (VSMCs), although synergistic enhancement of iNOS gene expression has been known to require multiple cytokines such as IL-1 β , tumor necrosis factor- α (TNF- α), IFN- γ and LPS, recent works have shown that IL-1 β alone is sufficient to induce the expression of iNOS gene in VSMCs.⁵⁻⁷⁾ IL-1 β is multifunctional cytokine, affecting nearly every cell type, and sometimes acts in concert with another proinflammatory cytokine such as TNF- α .⁸⁾ Specifically, IL-1 β is shown to initiate the expression of various genes including IFN, TNF and IL-1 β and to increase cell surface expression of IFN and TNF receptors.⁹⁾ Recent work has shown the potential role of IFN- γ in IL-1 β -mediated expression of iNOS gene by hepatocytes.¹⁰⁾ The importance of IFN- γ in the expression of iNOS gene by VSMCs has been previously demonstrated in several reports, indicating that IFN- γ is required for the maximal induction of iNOS gene expression in response to TNF- α and/or IL-1 β . Although investigators have emphasized the major role of IL-1 β in the expression of iNOS gene by VSMCs, they did not demonstrate a potential role of IFN- γ in the IL-1 β -mediated expression of iNOS gene by VSMCs.

The upstream portion of the murine iNOS promoter contains an enhancer region binding with several transcription factors in responses to IFN- γ . These sites include a gamma activated site element and interferon regulatory factor-1 (IRF-1) response element, which mediates binding by IRF-1.^{11,12)} This promoter region is necessary for full synergistic effects of IFN- γ on the induction of iNOS gene expression. Recently, Spink et al.¹³⁾ showed that IRF-1 is essential for maximal induction of

iNOS gene expression by IL-1 β and IFN- γ in VSMCs.

On the basis of these considerations, this study hypothesized that the IL-1 β -induced expression of iNOS gene in the VSMCs is dependent on the endogenous IFN- γ synthesis and mediated through the activation of IRF-1. This study determined the role of IFN- γ in the IL-1 β -induced expression of iNOS gene by using antisense IFN- γ oligodeoxynucleotide. I defined the contributions of IRF-1 activated by IFN- γ in addition to NF- κ B in the IL-1 β -induced expression of iNOS gene.

Materials and Methods

1. Materials

Rat recombinant IL-1 β and rat recombinant IFN- γ were obtained from R&D Systems (Minneapolis, MN). Taq polymerase and reverse transcriptase (Superscript) were purchased from Gibco BRL (Gaithersburg, MD). Anti-murine macrophage iNOS polyclonal antibody and polyclonal antibody against p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-rat IFN- γ monoclonal antibody was obtained from Pharmingen (San Diego, CA).

Mouse anti- α -actin and anti-mouse immunoglobulin G fluorescein isothiocyanate (FITC) conjugate were obtained from Sigma Chemical (St. Louis, MO). All other reagents for cell culture were purchased from Life Technologies (Gaithersburg, MD).

2. Cell cultures

VSMCs were isolated from rat thoracic aorta by enzymatic dissociation as described in elsewhere.⁵⁾ Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2mM glutamine, 100U/mL penicillin, 100 μ g/ml streptomycin, in an incubator at 37°C in 95% humidified air and 5% CO₂. They were passaged twice a week by harvesting with trypsin/EDTA and seeding in 10cm dishes. The identity of

the VSMCs was confirmed by indirect immunofluorescent staining for α -actin, by using mouse anti- α -actin antibody and anti-mouse IgG-FITC conjugate (Sigma Chemical Co, St. Louis, MO). The purity of the VSMCs cultures was routinely more than 99.0%. Cells at passages 7 to 15 were used for experiments. Cells were seeded on a 12-well plates or 60mm-culture dishes at 2×10^4 cells/cm² for transfection experiments, and allowed to grow in DMEM containing 10% serum. After reaching 80% confluent state, the cells were used for experiments.

3. Transfection of oligodeoxynucleotides to VSMCs

Phosphorothioate-modified oligodeoxynucleotides (ODNs) were designed to block initiation of target mRNA translation. The sequence of ODNs used as antisense (AS), scrambled antisense (Scr), and sense (S) DNA for IFN- γ were as follows: AS, 5'-TTCAATGAGTGTGCCTTGGCA GTA-3'; Scr, T-TCTAGAATGGTTGCTCTGGGCATA-3'; S, 5'-TACICCCAAECCACACICATTAGAA-3'. ODNs were transfected into the cells using LipofectAMINE reagent (Life Technologies, Gaithersburg, MD), 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleoyloxy-N-[2 (sperminecarboxyamino)ethyl]-N, Ndimethylpropanaminiumtrifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE). On the day of transfection, the medium was changed to fresh medium and incubated for 1 hour at 37°C. VSMCs were transfected with each ODNs (1 μ M) complexed with 2 μ g/mL of LipofectAMINE reagent according to the manufacturer's instructions and incubated in serum free Opti-MEM medium for 6 hours. Thereafter, cells were grown in DMEM medium containing 10% FBS for 18hours and treated with IL-1 β .

4. Measurement of nitrite Production

Nitrite concentrations were measured by mixing 100 μ L of culture medium with 100 μ L of Griess reagent as described previously.¹⁴⁾ The absorbance at 540nm was measured using Titertek Multiscan, and nitrite

concentrations were calculated from a sodium nitrite standard curve.

5. Biological activity Of IFN- γ

The biological activity of IFN- γ was demonstrated by testing the effect of supernatants collected from the IL-1 β -treated VSMCs on the production of NO in RAW264.7 cells stimulated with LPS as described previously.¹⁵⁾ Briefly, RAW264.7 cells were preincubated on a 24-well plates (1×10^6 cells/cm²) in DMEM containing 10% FBS for 6 hours. At the time of testing, medium was replaced with 500 μ L of fresh medium and 500 μ L of supernatant collected from the IL-1 β -treated VSMCs was added in duplicate. Cells were treated with or without LPS (10ng/ml) for 24 hours and nitrite concentrations were determined

6. Measurement of IFN- γ

Supernatant levels of IFN- γ were measured by Endogen Rat Interferon gamma enzyme-linked immunosorbent assay kit. Stripwell plates were precoated with anti-rat IFN- γ monoclonal antibody. 50 μ L of sample diluent were added to each along with 50 μ L of sample or standard and incubated at room temperature for 1 hour. The wells were washed 3 times, and 100 μ L of biotinylated antibody reagent was added and incubated at room temperature for 30min. The wells were washed 3 times, and 100 μ L of prepared streptavidine-horser peroxidase (HRP) was added and incubated at room temperature for 30min. After 3 times washes, 100 μ L of premixed 3, 3', 5', 5'- tetramethylbenzidine substrate solution was added and incubated at room temperature for 30min. The reaction was stopped with 100 μ L of stop solution (0.18M sulfuric acid) the absorbance at 540nm was read on a microplate reader. The standard curve was generated using curve-fitting software and used to determine the amount of rat IFN- γ in an unknown sample.

7. mRNA extraction and reverse transcription- polymerase chain reaction (RT-PCR)

Total RNA was prepared from cultured VSMCs using RNAzol (Tel-Test 12 hours after IL-1 β treatment. RNA (1 μ g) from each sample was reverse-transcribed with Oligo-dT as the first-strand cDNA primer and Maloney murine leukemia virus reverse Transcriptase. PCR amplification was carried out for 30 cycles using Tag polymerase on a PCR thermal cycler. PCR products were visualized by electrophoresis through 1.5% agarose gel containing ethidium bromide. The sequences of primer pairs used to amplify rat iNOS, IFN- γ , and TNF- α were as for 5' - T A G A G G A A G A T C T G G C C A G G - 3' , 5'-TGGCCGACCTGATGTTGCCA-3' (254bp products); 5' - T A C T G C C A A G G C A C A C T C A T T G A A - 3' , 5'-GCACCCACTCCTTT: TCCGCTTCCT-3' (407bp products) ; 5'-ATGAGCACGGAAAGCATGATC-3' 5'-TACGGGCTTGTCACCTCGAGTT-3' (275bp products). The primer sequence: for α -actin mRNA, used to determine constitutive mRNA expression, were 5' - C A T C G T G G G C C G C T C T A G G C A C - 3' , 5' - C C G G C C A G C C A A G T C C A G A C G C - 3 (450bp products).

8. Western blotting

Total proteins were prepared in lysis buffer as described previously.⁴⁾ Samples containing 50 μ g of protein were resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and immunoblotted with polyclonal antibody against murine macrophage iNOS or monoclonal antibody against rat IFN- γ . Antibodies were detected by HRP-conjugated secondary antibody and visualized with an enhanced chemiluminescence detection kit (Amersham).

9. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared by the method of Staal et al.¹⁶⁾ A double-stranded oligonucleotide corresponding NF- κ B (5'-CCAACTGGGGACTCTCCCTTTGGGAACA-3'; or IRF-1 (5'-CACGTGCAATATTTTCACTTTTTCATAATG-3') was end-labeled with T4 polynucleotide kinase (Promega, Madison, WI) in a reaction containing 2 μ L Probe

(1.75pmole/ μ L), 1 μ L 10 \times T4 kinase buffer, 1 μ L (γ -32P) ATP (10mCi/mL), 5 μ L water, and 1 μ L T4 kinase (10U/ μ L). After incubation at 37 $^{\circ}$ C for 30min, the reaction was stopped by adding of 1 μ L 0.5M EDTA and then 89 μ L TE buffer. For each binding reaction, aliquots of the nuclear extract (8 μ g) were suspended in a final volume of 12.5 μ L by adding 2.5 μ L of 5 \times binding buffer [100mM HEPES (pH 7.9), 250mM KCl, 5mM MgCl₂, 0.5mM EDTA, 5mM DTT, 2mM PMSF, 1 μ g/mL Poly (dl-dC), 25% glycerol], appropriate amounts of water and 1 μ L of radiolabeled probe. Before the labeled probe was added, the mixture was incubated on ice for 10min. After the probe was added, the incubation continued at room temperature for 30min. Reactions were analyzed by electrophoresis on a 6% nondenatured polyacrylamide gel in 0.5x TBE, at 200V and 4 $^{\circ}$ C. Gel was dried and bands corresponding to complexes between Transcription factors and the labeled probe were detected by autoradiography. For competition experiments, 100-fold molar excess of competitor DNA was incubated in the mixture prior to the addition of nuclear extracts. For supershift, polyclonal antibodies against NF- κ B or IRF-1 were added to the extracts 10min before adding the labeled probe.

10. Statistics

Data are presented as means \pm SEM for the number of experiments. Multiple comparisons were evaluated by Student's t-test. Result with P < 0.05 were considered statistically significant.

Results

1. IL-1 β -induced synthesis of endogenous IFN- γ in rat VSMCs

VSMCs were exposed to IL-1 β (1-100ng/mL) for 24 hours and the NO production was measured by nitrite concentrations. The NO production was increased in a dose-dependent manner and showed maximal accumulation

(approximately $36 \pm 7.0 \mu\text{M}$) in the presence of 100 ng/mL of $\text{IL-1}\beta$. In equal to the NO production, the $\text{IFN-}\gamma$ synthesis was also increased in a dose-dependent manner (Fig. 1A).

The maxim $\text{IFN-}\gamma$ synthesis ($263 \pm 12.0 \text{ pg/mL}$) occurred in the presence of 100 ng/mL of $\text{IL-1}\beta$. In the presence of $\text{IL-1}\beta$ (50 ng/mL), the productions of NO and $\text{IFN-}\gamma$ were also increased in a time-dependent manner (Fig. 1B). To confirm the endogenous $\text{IFN-}\gamma$ synthesis, the expression of $\text{IFN-}\gamma$ gene was assessed by Western blot and RT-PCR (Fig. 1C). In the unstimulated cells, $\text{IFN-}\gamma$ gene was not detectable. In the $\text{IL-1}\beta$ -stimulated VSMCs, however, the expressions of $\text{IFN-}\gamma$ protein and mRNA were detected and markedly increased in the presence of $\text{IL-1}\beta$ (50 ng/mL). These results indicate that the exposure of the VSMCs to $\text{IL-1}\beta$ induces the expressions of $\text{IFN-}\gamma$ protein and mRNA as well as the NO production. Further study was performed to determine whether $\text{IL-1}\beta$ induces the production of TNF. But in the presence of $\text{IL-1}\beta$ ($1\text{-}100 \text{ ng/mL}$), the expression of TNF mRNA was not detectable as determined by RT-PCR (data not shown).

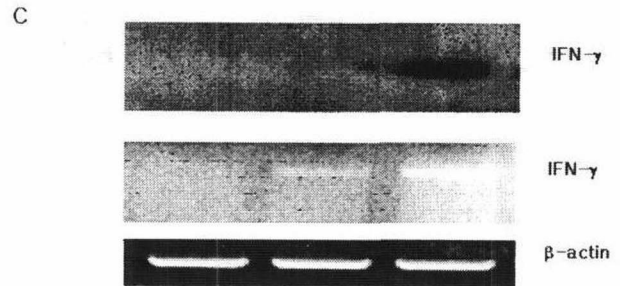
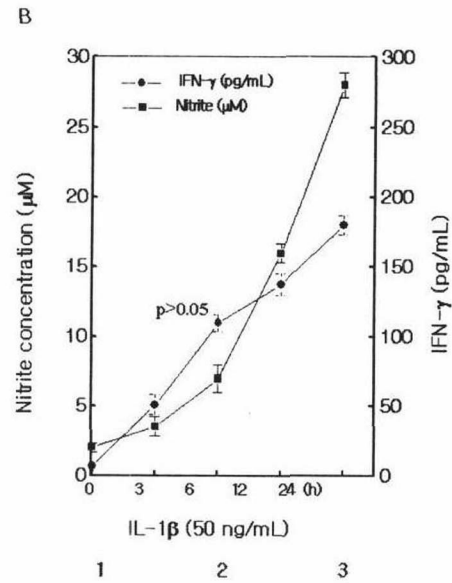
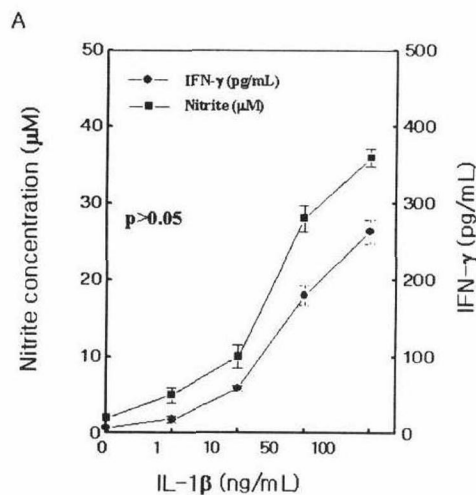


Fig. 1 (A) Dose- and (B) time dependent effects of $\text{IL-1}\beta$ on the productions of NO and $\text{IFN-}\gamma$ in the VSMCs. Cells were treated with different concentrations ($1\text{-}100 \text{ ng/mL}$) of $\text{IL-1}\beta$ for 24 hours and then nitrate accumulation and $\text{IFN-}\gamma$ concentration were measured. Data are means \pm SEM of triplicate experiments. (C) Effects of $\text{IL-1}\beta$ on expressions of $\text{IFN-}\gamma$ protein and mRNA in VSMCs protein ($50 \mu\text{g}$) of cytosolic fraction was separated in 10% SDS-PAGE and transferred onto nitrocellulose membrane. $\text{IFN-}\gamma$ protein was determined by western blot with monoclonal antibody against rat $\text{IFN-}\gamma$. Total RNA was isolated and then RT-PCR with $\text{IFN-}\gamma$ specific primers was performed as described in methods. Lane 1, unstimulated cells; Lane 2, $\text{IL-1}\beta$ (10 ng/mL); Lane 3, $\text{IL-1}\beta$ (50 ng/mL). Data are representatives of triplicate experiments.

2. Effects of culture supernatant derived from $\text{IL-1}\beta$ -stimulated VSMCs on the NO production in RAW264.7 cells treated with LPS

It has been well established that the expression of iNOS

gene in macrophages is maximally induced by a combination of LPS and IFN- γ . To further confirm the cellular synthesis of IFN- γ , the biological activity of IFN- γ was assessed by testing the effects of supernatants collected from the IL-1 β -stimulated VSMCs on the NO production by the RAW264.7 cells in the presence or absence of LPS (Fig. 2A).

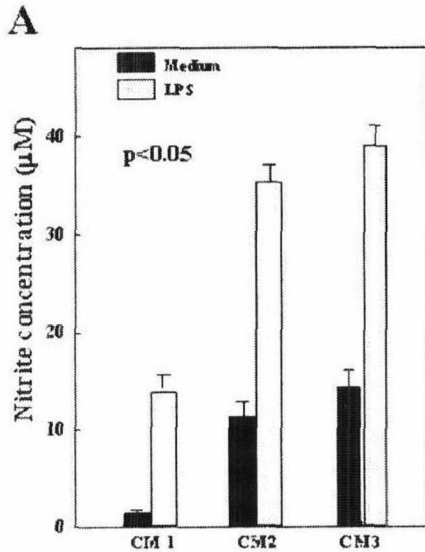


Fig. 2 (A) Effects of culture supernatants derived from IL-1 β stimulated VSMCs and (B) supplemented with neutralizing anti IFN- γ antibody on the NO production in RAW264.7 stimulated with LPS. RAW264.7 (CM1), or containing 10ng/mL of IL-1 β (CM2) or 50ng/mL of IL-1 β (CM3) in the presence or absence of LPS (10ng/mL) for 24 hours, and then nitrate accumulation was measured. The transferred conditioned medium (CM) derived from VSMC cultures treated with IL-1 β (50ng/mL) for 24 hours were additionally supplemented with either control IgG or anti IFN- γ antibody and incubated with or without LPS (10ng/mL) for 24 hours.

Treatment of RAW264.7 cells with supernatants from the unstimulated VSMCs culture did not induce the NO production. However, addition of supernatants from the IL-1 β -stimulated VSMCs to the RAW264.7 cells induced large amounts of NO production. In addition, the conditioned medium synergistically increased the NO production in the presence of LPS. In this system, the potential role of IFN- γ was further examined by using the neutralizing anti-IFN- γ antibody. The transferred

supernatants from VSMCs cultures treated with or without 50ng/mL of IL-1 β to the RAW264.7 cells were supplemented with control IgG or anti-IFN- γ antibody and then incubated for 24 hours in the presence or absence of LPS. Addition of control IgG had no effect on the NO production induced by conditioned medium containing IL-1 β in RAW264.7 cell treated with LPS. In contrast, these stimulatory effects of conditioned medium were blocked by addition of the neutralizing anti-IFN- γ antibody to the cells (Fig. 2B). But addition of the antibody against TNF- α had no effect on the production of NO (data not shown).

3. Effects Of antisense IFN- γ ODN OR the NO Production by the IL-1 β -stimulated VSMCs

VSMCs were transfected with each IFN- γ sense, scrambled, and antisense ODNs (2 μ M) complexed with lipofectAMINE reagent for 6 hours. After 18 hours further incubation with DMEM medium containing 10% FBS, VSMCs were treated with IL-1 β (50ng/mL) for 24 hours, and then the concentrations of IFN- γ in the culture medium were measured by enzyme-linked immunosorbent assay.

The addition of sense and scrambled antisense ODNs did not affect the IL-1 β -induced IFN- γ production. However, the addition of antisense ODN to the IL-1 β -stimulated cells inhibited the IFN- γ Production to 34 ± 6.0 pg/mL (Fig. 3A). To confirm the role of IFN- γ in the IL-1 β -induced NO production, nitrite accumulations were measured from supernatants. The addition of sense and scrambled antisense ODNs had no effect on the IL-1 β -induced NO production. However, treatment of the cells with antisense ODN inhibited the IL-1 β -induced NO production to 8.5 ± 3.5 μ M (Fig. 3B). Next study was performed to further confirm the specific action of antisense ODN to block the IFN- γ synthesis. As shown in figure 3C, the addition of sense ODN did not affect the IL-1 β -induced NO production. In the presence of IL-1 β /antisense ODN, the addition of IFN- γ (100U/mL)

restored the NO production. However, IFN- γ alone produced small amount of NO and synergized with IL-1 β to produce large amount of NO (Fig. 3C). These data suggest that antisense IFN- γ ODN inhibited the IFN- γ synthesis and did not affect the iNOS gene expression, and IFN- γ alone is not sufficient but necessary factor required for the maximal NO production in the IL-1 β -stimulated VSMCs.

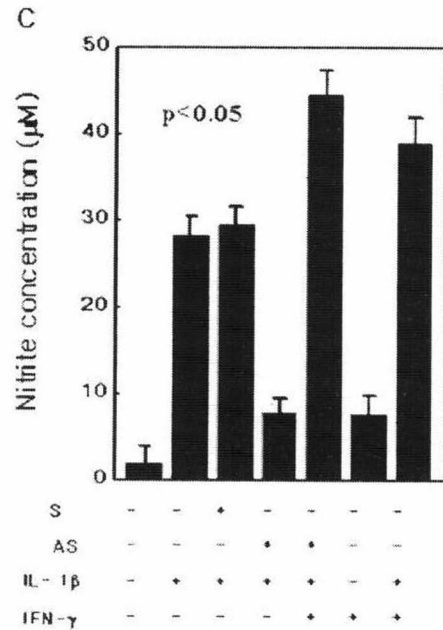
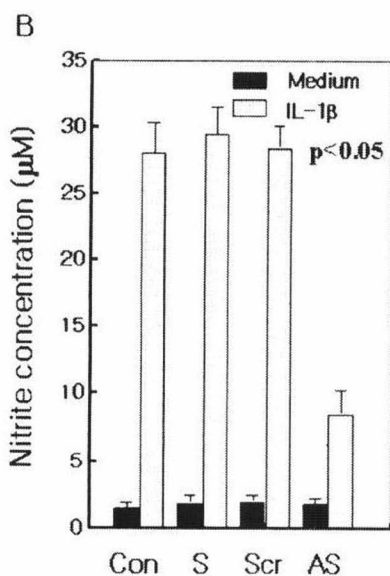
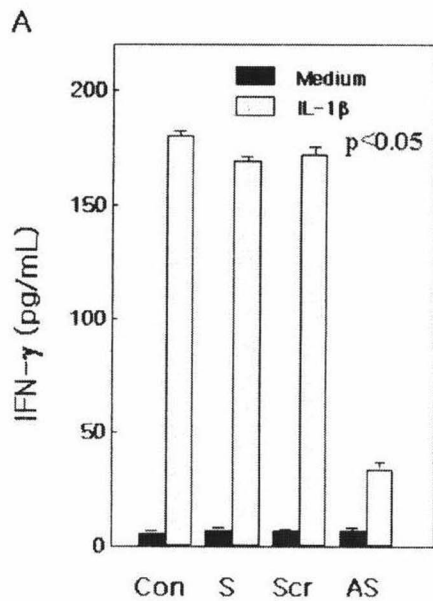


Fig. 3 Effects of antisense IFN- γ ODN on the IL-1 β -induced (A) IFN- γ synthesis and (B) NO production (C) Effects of exogenous IFN- γ on IL-1 β induced NO production by the VSMCs. Cells were transfected with sense, scrambled, and antisense ODNs (2 μ M) for IFN- γ as described in methods. The concentrations of IFN- γ and NO were determined by enzyme-linked immunosorbent assay, Griess reaction respectively. Data are means \pm SEM of triplicate experiment.

4. Effects Of antisense IFN- γ ODN on the iNOS gene expression by the IL-1 β -stimulated VSMCs

Immunoblot analysis was performed to determine the inhibition of iNOS gene expression by antisense ODN (Fig. 4A). In the presence of IL-1 β , the iNOS protein expression was markedly increased. The addition of sense and scrambled antisense ODNs did not affect the IL-1 β -induced iNOS protein expression. However, the IL-1 β -induced iNOS protein expression was inhibited by the treatment with antisense ODN. In the presence of IL-1 β /antisense ODN, the addition of IFN- γ (100U/mL) to the cells restored the iNOS protein synthesis completely. RT-PCR was performed to determine the effects of antisense ODN on the IL-1 β -induced iNOS mRNA

expression (Fig. 4B). In the presence of IL-1 β (50ng/mL), sense and scrambled antisense ODNs did not affect the IL-1 β -induced expressions of iNOS and IFN- γ mRNA. However, antisense ODN decreased the IL-1 β -induced expressions of iNOS and IFN- γ mRNA. To reconfirm the role of IFN- γ in the IL-1 β -induced NO production, immunoblot analysis was performed using monoclonal anti-rat IFN- γ antibody (Fig. 5). The IL-1 β -induced NO production and iNOS protein expression were blocked by anti-IFN- γ antibody ($\geq 20 \mu\text{g/mL}$). However, exogenous IFN- γ restored completely iNOS protein expression. These results suggest that IFN- γ is a necessary factor in the IL-1 β -induced iNOS gene expression.

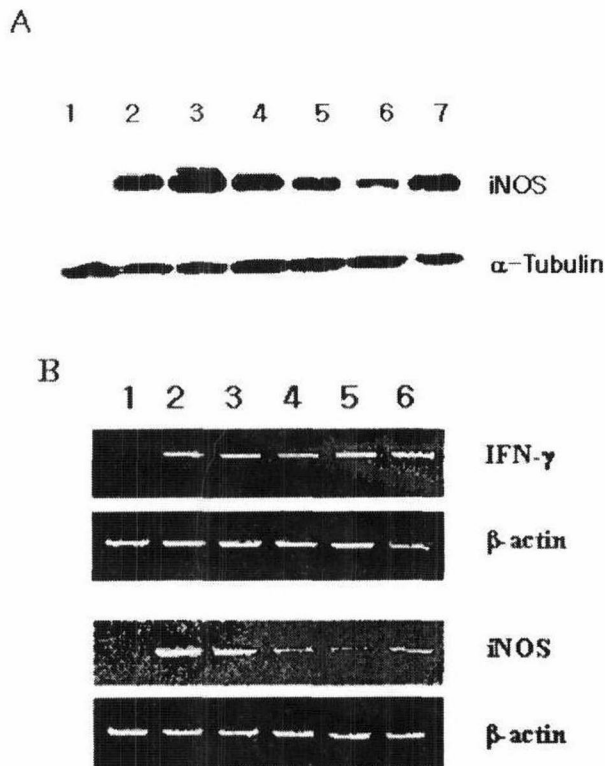


Fig 4 Effects of antisense IFN- γ ODN on the expressions of (A) iNOS protein and (B) IFN- γ mRNA and iNOS mRNA in the IL-1 β -stimulated VSMCs. Protein (50 μg) of cytosolic fraction was separated and transferred onto nitrocellulose membrane. iNOS protein was determined by western blot with monoclonal anti-iNOS antibody. Total RNA was isolated and then RT-PCR with iNOS specific primers was performed as described in methods. Lane 1, unstimulated cells : Lane 2,

IL-1 β (50ng/mL): Lane 3, IL-1 β /sense ODN (2 μM): Lane 4, IL-1 β /scrambled antisense ODN (2 μM): Lane 5, IL-1 β /antisense ODN (2 μM): Lane 6, IL-1 β /antisense ODN/IFN- γ (100U/mL). Data are representatives of triplicate experiment

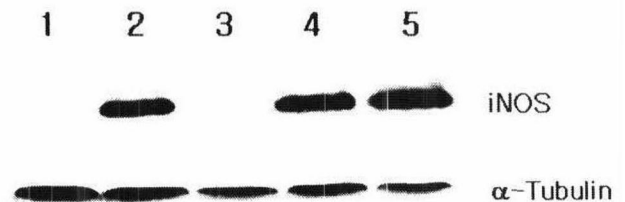


Fig 5 Effects of anti-IFN- γ antibody on the IL-1 β -induced iNOS protein expression in the VSMCs. iNOS protein was determined by western blot with monoclonal anti-iNOS antibody. Lane1, unstimulated cells: Lane2, IL-1 β (50ng/mL): Lane 3, IL-1 β /mouse IgG (1mg/mL): Lane 4, IL-1 β /anti-IFN- γ antibody (20 $\mu\text{g/mL}$): Lane 5, IL-1 β /anti-IFN- γ antibody/IFN- γ (100U/mL). Data are representatives of triplicate experiment.

5. Effects Of antisense IFN- γ ODN On the activations Of NF- κ B and IRF-1 in the IL-1 β -stimulated VSMCs

The activation of NF- κ B is reported to be required for the iNOS gene expression in the IL-1 β -stimulated VSMCs, and it is known that the activation of IRF-1 is mediated by IFN- γ .¹⁷⁾ To further establish the role of IFN- γ in the IL-1 β -induced iNOS gene expression, electrophoretic mobility shift assay was performed. As shown in figure 6A, the NF- κ B binding activity was present in nuclear extracts from the IL-1 β -stimulated VSMCs, but not from the unstimulated cells. Sense and antisense ODNs (2 μM) and IFN- γ (100U/mL) all had no effect on the NF- κ B binding activities induced by IL-1 β . IL-1 β -induced NF- κ B binding activity was inhibited by the addition of antibody to p65 subunit (Fig. 6A).

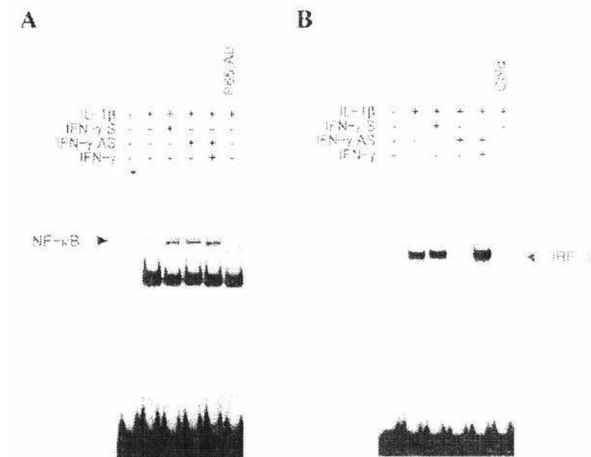


Fig. 6 Effects of antisense IFN- γ ODN on the (A) NF- κ B and (B) IRF-1 binding activities in the IL-1 β -stimulated VSMCs. Cells were transfected with sense and antisense ODNs for IFN- γ as described in methods. Cells were incubated with IL-1 β for 2 hours and nuclear extracts were prepared and analyzed for DNA-binding activity as described in Methods. Data are representatives of triplicate experiment.

In contrast, the IL-1 β -induced IRF-1 binding activity was dramatically inhibited by antisense ODN. As shown in figure 6B, the IRF-1 binding activities were detected in nuclear extract from the IL-1 β -stimulated VSMCs, but not from the unstimulated cells. Sense ODN did not affect the IRF-1 binding activity induced by IL-1 β . However, antisense ODN (2 μ M) strongly inhibited the IL-1 β -induced IRF-1 binding activity and the inhibition was fully recovered by the addition of IFN- γ . The IRF-1 binding activity was competitively inhibited by the addition of excess unlabeled probe, indicating the specific DNA/Protein binding (Fig. 6B). These results suggest that the IL-1 β -induced expression of iNOS gene in the VSMCs is regulated through the activations of NF- κ B and IRF-1, the latter of which via the endogenous IFN- γ synthesis.

Discussion

In the current study, this study have shown the expression of IFN- γ gene in the IL-1 β -stimulated VSMCs. The inhibition of IFN- γ gene expression by antisense IFN- γ ODN was associated with decreased NO production and iNOS gene expression in the IL-1 β -stimulated VSMCs. Similar results were observed in experiments using anti-IFN- γ antibody. Moreover, antisense ODN did not affect the activation of NF- κ B, but abolished the IL-1 β -induced IRF-1 binding activity. Blocking of IRF-1 activation by antisense IFN-ODN resulted in decreased iNOS gene expression. These evidences suggest that the IL-1 β -induced iNOS gene expression in the VSMCs is regulated through the activation of IRF-1 as well as NF- κ B, and endogenous IFN- γ synthesis is obligatory for the activation of IRF-1.

The expression of iNOS gene has demonstrated to be regulated at the level of transcription in response to proinflammatory mediators such as IL-1 β , TNF- α and IFN- γ . In most cases, maximal induction of iNOS gene expression requires a combination of two or more cytokines or a combination of one or nor cytokines plus LPS.^{18,19)} Previously, several studies of signaling pathway involved in cytokine-stimulated iNOS induction have shown that IL-1 β by itself is a sufficient stimulus in VSMCs.^{5-7,20)} These studies indicated that IL-1 β is primary activator for the iNOS gene expression by the VSMCs. In other cell systems such as macrophages, however, IFN- γ serves as a primary activator.^{11,12)} Recently, the importance of IFN- γ in the expression of iNOS gene by VSMCs has been demonstrated by Gross et al.²¹⁾ They showed that stimulants such as TNF- α , IL-1 β , and LPS induce the iNOS gene expression in the VSMCs by the mechanism that is synergistic with IFN- γ . Spink et al.¹³⁾ showed that IFN- γ is required for the maximal induction of iNOS gene expression in response to TNF- α and/or IL-1 β . They have shown that IFN- γ synergizes with cytokines to induce iNOS gene expression

in the VSMCs. However the mechanisms by which IL-1 β alone induce iNOS gene express and synergizes with IFN- γ and/or TNF- α to result in maximal NO production are unknown. In the present work, for the first time show that the IL-1 β -induced iNOS gene expression is dependent on the endogenous IFN- γ synthesis in the VSMCs. It characterized the expression of IFN- γ gene in the IL-1 β -stimulated VSMCs and defined the role of IFN- γ in the IL-1 β -induced iNOS gene expression by using antisense IFN- γ ODN and anti-IFN- γ antibody. The murine iNOS promoter region contains several binding sites for transcriptional factors, including NF- κ B, IRF-1, Oct-1, and Stat 1 α .²²⁾ Early studies have focused on the role of NF- κ B activation, which mediated the expression of iNOS gene in the LPS-stimulated macrophages as well as the IL-1 β -stimulated VSMCs.^{23,24)} Moreover, recent evidences have shown that the synergistic effect of IFN- γ on the LPS-induced iNOS gene expression is mediated by the activation of IRF-1.^{25,26)} In my experiments employing rat primary VSMCs, IL-1 β -induced NF- κ B binding activity was not affected by antisense IFN- γ ODN. However, the IL-1 β -induced iNOS gene expression was decreased by antisense IFN- γ ODN. These findings suggest that additional regulatory factors as well as NF- κ B is required for the optimal induction of iNOS gene expression in the IL-1 β -stimulated VSMCs.

IRF-1 is a transcriptional factor that was first identified as important in the virus-induced activation of the IFN- γ gene.²⁷⁾ IRF-1 is generally present only at low levels within resting cells; but after treatment with IFN, the transcription of IRF-1 gene is increased dramatically.²⁸⁾ The production of IRF-1 is induced by other cytokines such as IL-1 β , IL-6 and TNF- α , viral infection, and prolactin. Kamijo et al. have shown that IRF-1 binding is required for the synergistic effects of IFN- γ on the induction of iNOS gene expression.^{25,26)} Spink et al.²⁹⁾ have shown that transcriptional factors NF- κ B and IRF-1 are essential for the maximal induction of iNOS gene expression by IL-1 β , IFN- γ and TNF- α in the VSMCs. However, it has not

been demonstrated that the IRF activation in VSMCs is involved in IL-1 β -induced iNOS gene expression. Using a model of rat primary VSMCs, we showed that the IL-1 β -induced iNOS gene expression is regulated by the activations of IRF-1 as well as NF- κ B. Antisense IFN- γ ODN did not affect the activation of NF- κ B but abolished IRF-1 binding activity. Blocking of IRF-1 activation by antisense ODN was associated with decreased iNOS gene expression. These results suggest that IL-1 β induces endogenous IFN- γ synthesis to result in the induction of iNOS gene expression through the activations of IRF-1 as well as NF- κ B.

IL-1 β is a well-characterized pro-inflammatory cytokine released primarily by activated monocytes in response to a wide variety of infectious stimuli.⁹⁾ IL-1 β release in vivo is thought to potentiate many of the systemic effects of septic shock.³⁰⁾ Recent evidence supports a role of IL-1 β in the etiology of gastric cancer.³¹⁾ This report describes that the pro-inflammatory effects of IL-1 β may exacerbate mucosal damage and create an environment that makes individuals infected with H. Pylori more susceptible to carcinogenesis. Considering the associations of IL-1 β with inflammation or cancer, it will be critical to characterize properties of cytokines responsible for immune response. In particular, understanding the mechanism associated with the induction of iNOS gene in VSMCs may be also worthy of attention to prevent the deleterious hypotension associated with septic shock in the immune system.

Conclusion

This study have identified the NO production and the endogenous IFN- γ synthesis in the IL-1 β -stimulated VSMCs. In the experiments using antisense IFN- γ ODN and anti-IFN- γ antibody, I confirmed that IFN- γ Plays a Pivotal role in mediating the IL-1 β -induced iNOS gene expression. I also demonstrated that the IL-1 β -induced

iNOS gene expression is regulated through the activations of IRF-1 as well as NF- κ B. These results suggest that the IL-1 β -induced iNOS gene expression in the VSMCs is regulated through the activations of both NF- κ B and IRF-1 and endogenous IFN- γ synthesis is obligatory.

REFERENCES

- Nathan C: Nitric oxide as a secretory product of mammalian cells. *FASEB J* 12:3051-3064, 1992
- Stuehr DJ, Marletta MA: Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. *J Immunol* 139:518-525, 1987
- Nathan C, Xie QW: Regulation of biosynthesis of nitric oxide. *J Biol Chem* 269:13725-13728, 1994
- Kilbourn RG, and Belloni P: Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. *J Natl Cancer Inst* 82:772-776, 1990
- Beasley D, Schwartz JH, Brenne B: Interleukin 1 induces prolonged L-arginine-dependent cyclic guanosine monophosphate and nitrite production in rat vascular smooth muscle cells. *J Clin Invest* 87:602-608, 1991
- Durante W, Schini VB, Scott-Burden T, Junquero DC, Kroll MH, Vanhoutte PM, Schafer AI: Platelet inhibition by an L-arginine-derived substance released by IL-1 β beta-treated vascular smooth muscle cells. *Am J Physiol* 261:H2024-H2030, 1991
- Kanno K, Hirata Y, Imai T, Marumo F: Induction of nitric oxide synthase gene by interleukin in vascular smooth muscle cells. *Hypertension* 22:34-39, 1993
- Dinarello CA: Interleukin-1, Cytokine Growth Factor Rev 8:253-265, 1997
- Dinarello CA: Biologic basis for interleukin-1 in disease. *Blood* 87:2095-2147, 1996
- Schroeder RA, Gu JS, Kuo PC: Interleukin 1beta-stimulated production of nitric oxide in rat hepatocytes is mediated through endogenous synthesis of interferon gamma. *Hepatology* 27:711-719, 1998
- Xie QW, Whisnant R, Nathan C: Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. *J Exp Med* 177:1779-1784, 1993
- Lowenstein C J, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, Murphy WJ: Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon gamma and lipopolysaccharide. *Proc Natl Acad Sci* 90:9730-9734, 1993
- Spink J, Evans T: Binding of the transcription factor interferon regulatory factor-1 to the inducible nitric-oxide synthase promoter. *J Biol Chem* 272:24417-24425, 1997
- Yoo JC, Pae HO, Choi BM, Kim WI, Kim JD, Kim YM, Chung HT: Ionizing radiation potentiates the induction of nitric oxide synthase by IFN- γ and lipopolysaccharide in bnl cl.2 murine embryonic liver cells: role of hydrogen peroxide. *Free Radic Biol Med* 28:390-396, 2000
- Kim YM, Son KH: A nitric oxide production bioassay for interferon- γ . *J Immunol Meth* 198:203-209, 1996
- Staal FJ, Roederer M, Herzenberg LA: Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. *Proc Natl Acad Sci* 87:9943-9947, 1990
- Fujita T, Kimura Y, Miyamoto M, Barsoumian EL, Taniguchi T: Induction of endogenous IFN- γ alpha and IFN- γ beta genes by a regulatory transcription factor, IRF-1. *Nature* 337:270-272, 1989
- Beasley D, Eldridge M: Interleukin-1 beta and tumor necrosis factor-alpha synergistically induce NO synthase in rat vascular smooth muscle cells. *Am J Physiol* 266:R1197-R1203, 1994
- Busse R, Mulsch A: Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. *FEBS Lett* 275:87-90, 1990
- Wong HR, Funder JD, Wasserloos K, Lowenstein DJ, Geller DA, Billiar TR, Pitt BR, Davies P: Transcriptional regulation of iNOS by IL-1 β beta in cultured rat pulmonary artery smooth muscle cells. *Am J Physiol* 271:L166-L171, 1996
- Gross SS, Levi R: Tetrahydrobiopterin synthesis: an absolute requirement for cytokine-induced nitric oxide generation by vascular smooth muscle cells. *J Biol Chem* 267:25722-25729, 1992
- Xie QW, Kashiwabara Y, Nathan C: Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem* 269:4705-4708, 1994
- Hecker M, Preiss C, Schini-Kerth VB, Busse R: Antioxidants differentially affect nuclear factor kappa B-mediated nitric oxide synthase expression in vascular smooth muscle cells. *FEBS Lett* 380:224-228, 1996
- Schini-Keith V, Bara A, Mulsch A, Busse R: Pyrrolidine dithiocarbamate selectively prevents the expression of inducible nitric oxide synthase in rat aorta. *Eur J Pharmacol* 265:83-87, 1994
- Kamijo R, Harada H, Matsuyama T, Bosland M, Gerecitano J, Shapiro D, Le J, Koh SI, Kimura T, Green SJ: Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* 263:1612-1615, 1994
- Martin E, Nathan C, Xie QW: Role of interferon regulatory factor 1 in induction of nitric oxide synthase. *J Exp Med* 180:977-984, 1994
- Fujita T, Sakakibara J, Sudo Y, Miyamoto M, Kimura Y, Taniguchi T: Evidence for a nuclear factor(s), IRF-1, mediating induction and silencing properties to human IFN- γ beta gene regulatory elements. *EMBO J* 7:3397-3405, 1988
- Fujita T, Reis LF, Watanabe N, Kimura Y, Taniguchi T,

- Vicek J: Induction of the transcription factor IRF-1 and interferon-beta mRNAs by cytokines and activators of second-messenger pathways. *Proc Natl Acad Sci* 86:9936-9940, 1989
29. Spink J, Cohen J, Evans TJ: The cytokine responsive vascular smooth muscle cell enhancer of inducible nitric oxide synthase. Activation by nuclear factor-kappa. *B J Biol Chem* 270:29541, 1995
30. Cannon JG, Tompkins RG, Gelfand JA, Michie HR, Stanford G, van der Meer JW, Endres S, Lonnemann G, Corsetti J, Chernow B: Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. *J Infect Dis* 161(1):79-84, 1990
31. E-Omar EM, Carrington M, Chow WH, McColl KE, Bream JF, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni Jr. JF, Rabkin CS: Interleukin-1 polymorphisms associated with increased risk of gastric cancer. *Nature* 404:398-402, 2000