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Discoidin domain receptor 1 functions in nitric oxide induction in macrophages

조선대학교 대학원

식품영양학과

박 현

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대식세포에서 일산화질소 유도에 대한 discoidin domain receptor 1의 기능

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지도교수 이 명 렬

이 논문을 이학석사학위신청 논문으로 제출함.

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조 선 대 학 교 대 학 원

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2005年 11 月 28 日

조선대학교 대학원

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대식세포에서 일산화질소 유도에 대한 discoidin domain receptor 1의 기능

박 현

Advisor: Professor Myung-Yul Lee Ph.D.
Department of Food and Nutrition Graduate School of Chosun University

Nitric oxide (NO)는 면역반응의 중요한 조절인자 중의 하나이며, 특히 대식세포에서 발생되는 NO는 세균과 같은 외부침입물질이나 종양에 대한 숙주의 방어기전에 중요한 역할을 한다. Tumor necrosis factor-α나 interferon-γ같은 사이토카인과 lipopoly - saccharide같은 박테리아 생산물에 의한 대식세포에서의 NO 생성에 대한 효과는 널리 알려졌으나, 콜라겐 같은 세포의 기질 단백질의 역할은 아직 명확히 밝혀지지 않았다. Discoidin domain receptor 1 (DDR1)은 사람 대식세포에 발현하는 non-integrin collagen receptor로서 DDR1이 활성화가 되면 대식세포의 분화와 cytokine/chemo-kine 생산을 촉진한다고 발표된 바 있다. 본 논문에서는 콜라겐에 의한 inducible NO synthase (iNOS)의 발현과 NO생산에 DDR1이 관여하는지를 마우스 대식세포를 이용하여 연구하였다. 대식세포에 콜라겐을 투여하면 iNOS의 발현이 농도 의존적으로 증가하였고, NO의 생성도 역시 증가되었다. 대식세포가 발현하고 있는 DDR1을 knock-down시킨 결과 콜라겐에 의한 NO의 생성이 감소하였으며, 또한 β1 integrin blocking antibody나 β1 integrin siRNA 어느 것도 콜라겐으로 유도되는 iNOS발현을 억제하지 않았다.

이 같은 결과는 콜라겐이 β1 integrin이 아닌 DDR1과의 상호작용에 의해 NO를 생성하고, 이 상호작용의 신호전달과정에 nuclear factor-κB, p38 및 c-jun N-terminal kinase mitogen-activated protein kinase가 매개되었다는 것을 의미한다. 그러므로 NO가 관여하는 염증성질환의 조절에 DDR1 신호체계의 중재가 효과적으로 사용될 수 있을 것으로 생각된다.

Ⅰ. INTRODUCTION

Discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase (RTK) with a unique extracellular domain homologous to discoidin 1 of *Dictyostelium discoideum* (Vogel et al., 1997; Vogel, 1999). Unlike other RTKs which are activated by growth factors, DDR1 is activated by the binding to its ligand collagen. DDR1 is constitutively expressed in epithelial cells of normal tissues, such as lung, kidney, colon, and brain, and also in tumor cells of epithelial origin, such as mammary, ovarian, and lung carcinomas. Activation of DDR1 functions in axon extension of granule neurons and Wnt-mediated mammary cell adhesion (Bhatt et al., 2000; Jonsson and Andersson, 2001). In addition, DDR1 protects p53-mediated apoptosis (Ongusaha et al., 2003). Primary vascular smooth muscle cells isolated from DDR1-null mice showed decreased proliferation, collagen attachment, and migration *in vitro* (Houet al., 2001; Hou et al., 2002).

Kim et al (Kim et al., 2002) previously reported that the expression of DDR1 could be induced in leukocytes, including neutrophils, monocytes, and lymphocytes, *in vitro*. *In vivo*, tissue-infiltrating mononuclear cells, especially macrophages, were positive for DDR1 mRNA. Activation of DDR1 with collagen facilitated the differentiation and chemokine production of macrophages, and maturation of monocyte-derived dendritic cells via activation of downstream signaling pathways involving nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase (MAPK) (Matsuyama et al., 2003a; Matsuyama et al., 2003b; Matsuyama et al., 2004). These previous observations have indicated that DDR1 plays a role in cell attachment, migration and proliferation; however, the underlying molecular mechanisms of DDR1 activation and its physiologic impact in immune cells remain unclear.

Macrophages contribute to the development of inflammatory responses by secreting an array of cytokines and chemokines in a tissue microenvironment.

Proinflammatory cytokines, such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α, are potent activators of macrophages and up-regulate the expression and production of cytokines and chemokines. At inflammatory sites, they also interact with the components of the extracellular matrix (ECM) through receptors, such as integrins, and are activated for increased release or production of cytokines/chemokines. For instance, activation of monocytes with collagen, the most abundant protein in the ECM, induced IL-1 release. Collagen-induced IL-1 release was only partially inhibited by an antibody (Ab) against α2β1 integrin, a classic cell-surface collagen receptor (Pacifici et al., 1991), suggesting that the presence of an alternative receptor involved in monocytes/macrophage-collagen interaction.

Nitric oxide (NO) regulates many physiological and pathophysiological processes as well as in the maintenance of neuronal communication, vascular regulation and immune system. Stimulants, such as LPS, cytokines, ECM proteins, induce macrophages to express inducible NO synthase (iNOS) that catalyzes the production of NO (Xie et al., 1994; Cho et al., 2002). This macrophage-derived NO is an important component of host defense against pathogens and tumor cells (Nussler and Billiar, 1993; Gross and Wolin, 1995; MacMicking et al., 1997). Previously the role of collagen on NO production was reported (Cho et al., 2002). Stimulation of macrophages with collagen induces iNOS expression and NO production; however the exact role of collagen onto iNOS expression and underlying signaling pathways linked to collagen receptors remained unidentified.

MAPKs are important serine/thrionine signaling kinases that are activated by phosphorylation and act as mediators of cellular responses to extracellular signals. There are three types of MAPKs important to mammalian cells. These include extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK (Seger and Krebs, 1995). NF-κB is a transcription factor that mediates the expression of a number of genes (Xie et al., 1994; Sha et al., 1995).

In resting state, NF-κB proteins are sequestered in the cytosol through interactions with an inhibitory protein IκB. Several stimuli, including cytokines, LPS, oxidant, and ECM, induce NF-κB activation. These signals induce phosphorylation and degradation of IκB protein, resulting in nuclear translocation of NF-κB and increased gene expression (May and Ghosh, 1998; Cho et al., 2002).

The p38 MAPK, JNK and NF-κB are known to play an important role in the production of NO (Chan et al., 1999; Chen and Wang, 1999). It was reported that the interaction of DDR1 with collagen activates p38 MAPK and NF-κB in PMA-induced differentiated THP-1 cells, a model of macrophages (Kim et al., 2002), and in monocyte-derived primary macrophages (Matsuyama et al., 2003b), led us to the hypothesis that activation of DDR1 may contribute collagen-induced NO production in macrophages. In the present study, I tested my hypothesis using murine macrophages. I present results demonstrate that collagen-activation of DDR1 but not α2β1 integrin mediates the up-regulation of iNOS expression and concomitant NO production in p38 and JNK MAPK, and NF-κB dependent manner. Thus, DDR1-collagen interaction up-regulates the production of NO by macrophages and it is likely to contribute to the development of inflammatory responses in a tissue microenvironment.

II. MATERIALS AND METHODS

Reagents

A soluble form of bovine collagen type I was purchased from Sigma (St. Louis, MO) and added to the culture. Concentration of endotoxin was less than 10 pg/ml according to a colorimetric Limulus ameobocyte lysate assay (Whittaker Bioproducts, Walkersville, MD). LPS (from E.coli 026:B6, Sigma) was dissolved in the medium. The stocks were aliquoted and stored at 20℃ until use. SB203580, PD98059 and SP600125 were from Calbiochem (La Jolla, CA). Rabbit polyclonal Abs against DDR1, iNOS, IκBα and p65 NF-κB were from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse mAb against phosphotyrosine (4G10) or β1-integrin blocking Ab were from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal Abs against phosphorylated or nonphosphorylated p38, JNK and ERK were from Cell Signaling Technology (Beverly, MA). Human IgG1-Fc mAb, DMEM, recombinant protein G-agarose (PGA), and TRIzol reagent were from Invitrogen (Gaithersburg, MD).

Cell cultures

BALB/c murine macrophage cells (J774A.1, ATCC TIB-67) were grown in DMEM medium supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% non-heat-inactivated fetal bovine serum in 5% CO_2 at 37°C.

Nitric oxide assay

The amount of stable nitrite, the end product of NO generation by activated macrophages, was determined by a colorimetric assay as previously described

(Kim et al., 2004). Briefly, 50 μl of culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrocholoride, 2.5% H_3PO_4), and incubated at room temperature for 10 min. The absorbance at 540 nm was read using a PowerWavex Microplate Scanning spectrophotometer (Bio-Tek Instrument, Winooski, VT). Nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

Analysis of mRNA expression

Total cellular RNA was isolated from macrophages following 6 h of treatment with collagen or other agents using TRIzol according to manufacturer's protocol. The first strand complimentary DNA (cDNA) was synthesized using Superscript II reverse transcriptase enzyme (Invitrogen). Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyze the expression of mRNA for iNOS, DDR1 and β-actin (internal control). The condition for reverse transcription and PCR steps were performed as previously reported (Kim et al., 2002) with the exception of primer sets. Sense and antisense primers used were 5'CACCTTGGAGTTCACCCAGT3' and 5'ACCACTCGTACTTGGGATGC3' for iNOS, 5'GCTCCTGCTGCTCATCATTG3' and 5'TAATGGGGGACGCTGTTCTG3' for DDR1, 5'ATGGATGACGATATCGCT3' and 5'ATGAGGTAGTCTGTCAGGT3' for β-actin. Optimization of cycle number was performed to ensure that product accumulation was in a linear range. The number of cycle was 26-29 cycles based on preliminary trials. Amplified products were mixed with 10 DNA dye (5 mg/ml bromophenol blue, 50% glycerol, 100 mM Tris, 20 mM NaCl, 1 mM EDTA) and separated by electrophoresis on 2% agarose gels containing ethidium bromide. The gels were documented using a Kodak DC 290 digital camera and digitized using UN-SCAN-IT software (Silk Scientific, Orem, UT).

Western blot analysis

Each sample was mixed with double-strength sample buffer, boiled for 10 min, electrophoresed on precast 8-12% polyacrylamide gels by SDS-PAGE, and proteins were transferred to nitrocellulose membrane. Membranes were incubated with antibodies against IκBα, NF-κB (p65) (Santa Cruz Biotech, Santa Cruz, CA), or activated or non-activated form of each MAPK (Cell Signaling, Beverly, MA), followed by an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) (Cell signaling). HRP activity was visualized by enhanced chemiluminescence detection kit (Amersham Pharmacia, Piscataway, NJ).

Nuclear protein extraction

Nuclear extract was prepared as described elsewhere. Briefly, after cell activation for the times indicated, cells were washed in 1 ml of ice-cold PBS, centrifuged at 1,200 rpm for 5 min, resuspended in 400 μl of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000 g for 30 s. Pelleted nuclei were gently resuspended in 50 μl of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed and centrifuged at 15,000 g for 5 min at 4℃. Aliquots of supernatants that contained nuclear proteins were frozen in liquid nitrogen and stored at -70℃.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein (10 μg) was incubated for 20 min at room temperature with 20 μg of bovine serum albumin, 2 μg of poly (dI-dC) (Pharmacia, Uppsala, Sweden), 2 μl of buffer C (20 mM HEPES/KOH, 20% glycerol, 100 mM KCl, 0.5 mM PMSF, pH 7.9), 4 μl of buffer F (20% ficoll-400, 100 mM HEPES/KOH, 300 mM KCl, 10

mM DTT, 0.5 mM PMSF, pH 7.9), and 20,000 cpm of a 32P-labeled probe encoding the consensus sequence for NF-κB binding (5'-CAG AGG GGA CTT TCC GAG AG-3') in a final volume of 20 μl. DNA-protein complexes were resolved in a native 4% polyacrylamide gel at 160 V for 1.5 h, dried, and visualized (with autography using a Fuji x-ray film).

Immunofluorescence

The cells were fixed and permeabilized in 1:1 cold methanol: acetone for 30 min, washed twice with phosphate buffered saline (PBS), and incubated for 50 min with 1% normal goat serum in PBS containing 0.1% Triton X-100 to block non-specific staining. A mouse p65 NF-κB Ab diluted in PBS containing 2 mg/ml fatty acid-free bovine serum albumin (Sigma) and 0.1% Triton X-100 were added to the slides and incubated for 1 h at 37℃ in a humidified chamber. The slide was washed with PBS followed by 1 h incubation with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary Abs (Molecular Probes, Eugene, OR). The slides were washed, and mounted by cover slips and viewed using an Olympus IX70 FV300 confocal microscope (Ina, Japan) equipped with appropriate optics.

Luciferase activity assay

NF-κB luciferase reporter gene constructs (pNF-κB-LUC, plasmid containing NF-κB binding site; STANTAGEN, Grand Island, NY) were transiently transfected to J774A.1 cells using Lipofectamine (Invitrogen). Cells were allowed to recover at 37℃ for 24 h and subsequently were stimulated as indicated. For luciferase activity assay, cell lysates were prepared and assay for luciferase activity using Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

RNA interference

siRNA specific for mouse β1 integrin and control (scrambled) were purchased from Santa Cruz Biotechnology. J774 cells were cultured at a density of 3×10^6 cells/ml in complete medium in six-well plates for overnight. Cells were washed three times with PBS and transfected with the siRNA at the final concentration of 10 μM using Lipofectamine reagent according to the manufacturer's protocol. After 30 h incubation, cells were rinsed with PBS, treated with collagen, and incubated for the indicated time.

Plasmid construction

The expression construct for mDDR1-Fc was a kindly gift from Dr. Tomoda (Rockefeller University, New York, NY). Construction of mammalian expression vector for the mDDR1-Fc and ssFc-His6 fusion protein were previous described (Bhatt et al., 2000). J774 macrophages were transfected with a vector with or without insert, using Lipofectamine reagent. Eighteen hours after transfection, cells were washed with PBS, and stimulated with collagen for iNOS gene expression and NO production assay.

Statistical analysis

All statistical analyses were performed using SAS statistical software (SAS Institute, Cary, NC). Effects of treatment were analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test. A value of $p < 0.05$ was used to indicate significant differences.

Ⅲ. RESULTS

Collagen induces NO production and iNOS expression in J774 murine macrophages

To examine whether collagen induces NO production in macrophages, I first treated J774 murine macrophage cells with bovine type I collagen. As shown in Figure 1A, collagen dose-dependently increased NO production over the basal level at 24 h. The amount of NO produced with 50 μg/ml of collagen was similar to that produced with 30 ng/ml of LPS. When the cells were exposed to a combination of collagen and LPS, NO production was additively increased. From these results, I hypothesized that the mechanistic basis for the collagen-induced NO production is different from that for LPS-induced NO production. Collagen (50 μg/ml) stimulated the production of NO up to 96 h following treatment as shown in Figure 1B. The expression of iNOS mRNA and protein were also examined by RT-PCR (Fig. 1C) and Western blot (Fig. 1D). Both iNOS mRNA and protein were dose-dependently increased following 6 and 24 h treatment with collagen, respectively.

Collagen induces degradation of IκBα and translocation of NF-κB

Because it has been well known that the activation of iNOS is dependent on IκB α and NF-κB activation in LPS-stimulated macrophages (Xie et al., 1994; Cho et al., 2002), I next evaluated the effect of collagen on the activation of NF-κB. The level of IκBα rapidly decreased after treatment of cells with collagen, probably due to its degradation, and then returned to the original levels (Fig. 2A). Collagen treatment also induced nuclear translocation of p65 without altering the level of cytoplasmic p65. Exposure of cells to collagen also resulted in intensified nuclear staining for NF-κB, compared with the cytosolic staining in untreated cells (Fig. 2B). Collagen-induced activation of NF-κB was also confirmed by EMSA (Fig.

2C). Finally, I investigated whether collagen increases NF-κB-dependent gene transcription in J774 cells. To this end, J774 cells were transiently transfected with a NF-κB-luciferase reporter construct or the empty vector. Exposure of cells to collagen for 12 h showed significant increase of luciferase activity in the cells transfected with the NF-κB-luciferase reporter construct (Fig. 2D).

DDR1 is expressed in macrophages and activated by collagen

Cho et al (Cho et al., 2002) previously demonstrated that type I collagen induced iNOS expression and concomitant NO production in murine primary macrophages and emphasized the role of ECM, especially collagen, in the process. However, the exact mechanisms mediating collagen-induced NO production are still not clear. Several types of collagen serve as ligands for DDR1 and induce dimerization and subsequent transphosphorylation of DDR1 (Vogel et al., 1997; Vogel et al., 2000). I first examined the expression of DDR1 in J774 cells. As shown in Figure 3, DDR1 is constitutively expressed in J774 cells. Cells were incubated on uncoated tissue culture plates and then collagen was added to activate DDR1. DDR1 was immunoprecipitated with anti-DDR1 Ab from cell lysates and the kinetics of DDR1 phosphorylation were analyzed. The level of phosphorylation reached a peak at 1-1.5 h and decreased at 2 h.

Collagen induces NO production dependently of DDR1, but independently of β1 integrins

To examine the contribution of β1 integrins, the classic collagen receptor, in collagen-induced NO production, we used an anti-β1 integrin blocking Ab. As shown in Figure 4, addition of the Ab did not affect either collagen-induced NO production (Fig. 4A) or iNOS protein production (Fig. 4B).

It is well established that receptor tyrosine kinases, including DDR1, lacking their intracellular catalytic domain function as dominant-negative proteins by

oligomerizing with endogenously expressed functional wild-type receptors and also by competing for ligand (Bhatt et al., 2000; Vogel et al., 2000). A DDR1-Fc fusion protein, which lacked the intracellular catalytic domain of the DDR1, was previously used by others to inhibit DDR1 activation (Bhatt et al., 2000). To obtain additional evidence that collagen-induced NO production was a direct effect on DDR1, I transfected J774 cells with a construct for either mDDR1-Fc or ssFc, a secretable form of control Fc protein (Bhatt et al., 2000). As shown in Figure 5A, mDDR1-Fc or ssFc was detected in the culture supernatant of the transfected cells. I next stimulated these cells with collagen and compared the expression iNOS and production of NO with that by control cells. The cells transfected with mDDR1-Fc expressed much lower level of iNOS and produced only a low level of NO in response to collagen in compared to untransfected cells (Fig. 5B, C, D). In contrast, the level of iNOS expression and NO production by the cells transfected with ssFc or vector only was similar to that by untransfected cells, confirming the role of DDR1 in collagen-induced NO production.

To further discriminate the functional role of DDR1 and β1 integrins in collagen-induced NO production, we inhibited the expression of β1 integrins in J774 cells by RNA interference. Transfection of cells with β1 integrin-siRNA reduced the level of β1 integrin by 90%, but transfection of cells with control (scrambled)-siRNA did not change the level of β1 integrin compared with WT (Fig. 6A). Cytotoxicity was assessed after knockdown to determine the concentration of siRNA was safe. The transfected cells did not show cytotoxicity measured by MTT assay (data not shown). Stimulation of cells transfected by siRNA with collagen still increased the production of iNOS, and concomitant NO production. (Fig. 6B, C). The degradation of IκBα (Fig. 6D) and NF-κB-dependent gene transcription was also not interfered by inhibition of β1 integrin (Fig. 6E). These results strongly indicated that collagen-induced NO production was mediated through DDR1, but not β1 integrins.

Collagen-induced NO production is mediated by p38 and JNK, but not ERK pathway

I next attempted to identify downstream signaling molecules that may play a role in collagen-induced iNOS expression. Since LPS activates MAPKs signal-transduction pathways and downstream iNOS gene expression (Seger and Krebs, 1995; Kim et al., 2004), I compared the effect of collagen on the activation of MAPKs with LPS by examining the phosphorylation of p38, JNK and ERK by Western blot. Stimulation of J774 cells with LPS resulted in increased phosphorylation of all three MAPKs, and this phosphorylation peaked at 0.5-1 h (Fig. 7A) and decreased at 1.5 h (data not shown). Stimulation of cells with collagen also increased phosphorylation of all three MAPKs. Next I added a blocking Ab against β1 integrin to examine whether collagen-induced MAPKs activation was mediated by β1 integrin. Interestingly, pretreatment of anti-β1 integrin blocking Ab had little effect on collagen-induced p38 and JNK activation. However, the collagen-induced activation of ERK was dramatically decreased by anti-β1 integrin blocking Ab. These data suggest that both p38 and JNK were activated via DDR1 but ERK was activated via β1 integrins. To confirm our hypothesis, I inhibited the expression of β1 integrin using siRNA. Phosphorylation of p38 and JNK were not altered by inhibition of β1 integrin after collagen stimulation. However, collagen-induced activation of ERK was no longer detected after inhibition of β1 integrin expression (Fig. 7B). Finally, I used pharmacological inhibitors for NF-κB, p38, JNK, and ERK and evaluated the effects on collagen-induced NO production. As shown in Figure 8, specific inhibition of NF-κB, p38, JNK, and ERK resulted in a marked reduction in collagen-induced NO production in wild-type cells. However, in case of β1 integrin-siRNA transfected cells, treatment of a specific inhibitor of ERK, PD98059, did not decrease the collagen-induced NO production, although inhibition of NF-κB, p38, and JNK with specific inhibitor showed significant inhibition of collagen-induced NO production.

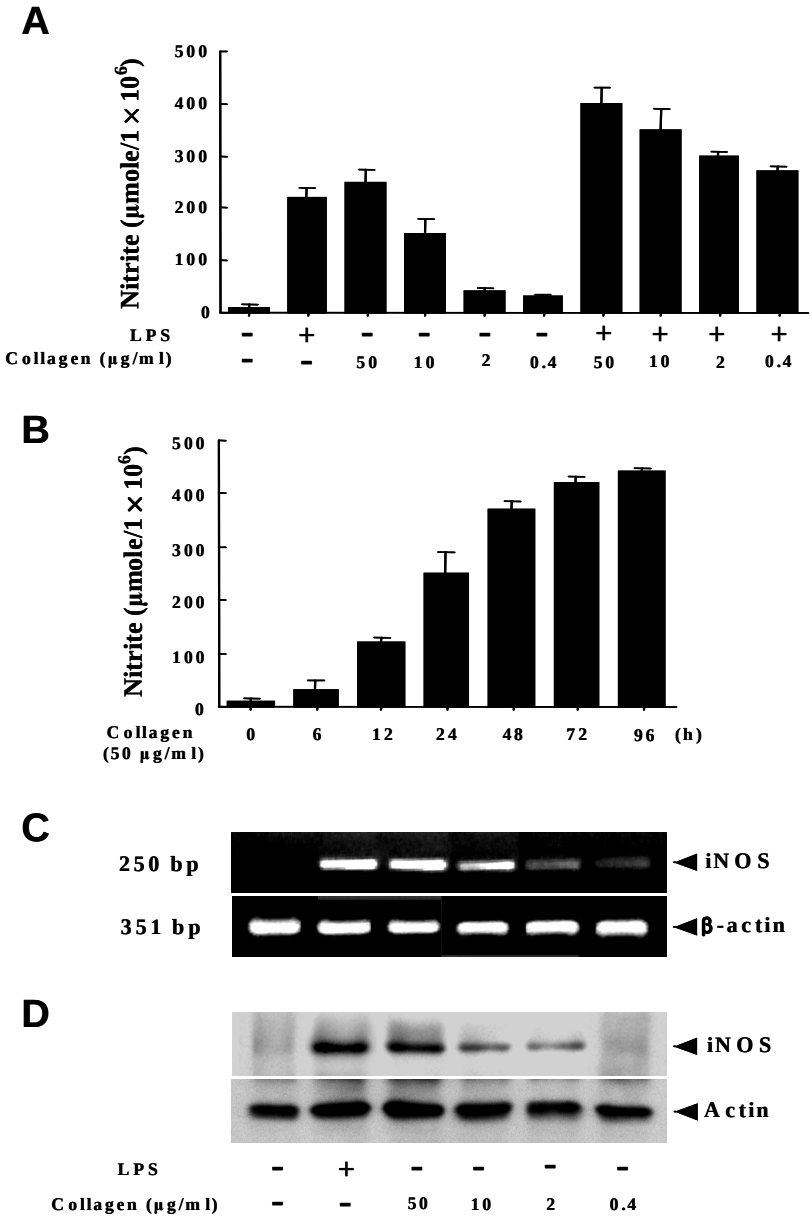

A
Nitrite (μmole/1 × 10^6)
500
400
300
200
100
0
LPS - + - - - - + + + +
Collagen (μg/ml) - - 50 10 2 0.4 50 10 2 0.4
B
Nitrite (μmole/1 × 10^6)
500
400
300
200
100
0
Collagen (50 μg/ml) 0 6 12 24 48 72 96 (h)
C
250 bp
351 bp
iNOS
β-actin
D
iNOS
Actin
LPS - + - - - -
Collagen (μg/ml) - - 50 10 2 0.4

Figure 1. Up-regulation of NO production and iNOS expression by collagen stimulation in J774 macrophages. Cells (1×10^6 cells/ml) were activated with collagen and/or LPS (30 ng/ml). A) After 24 h of treatment, NO in the culture media was assayed. B) Culture media was removed at 6, 12, 24, 48, and 96 h following collagen treatment and NO was determined. C) After 6 h of collagen and/or LPS (30 ng/ml) treatment iNOS gene expression was assayed by RT-PCR. D) After 24 h of collagen and/or LPS (30 ng/ml) treatment iNOS protein production was assayed by Western blot Each bar represents the mean±SEM (n=4) of two independent experiments.

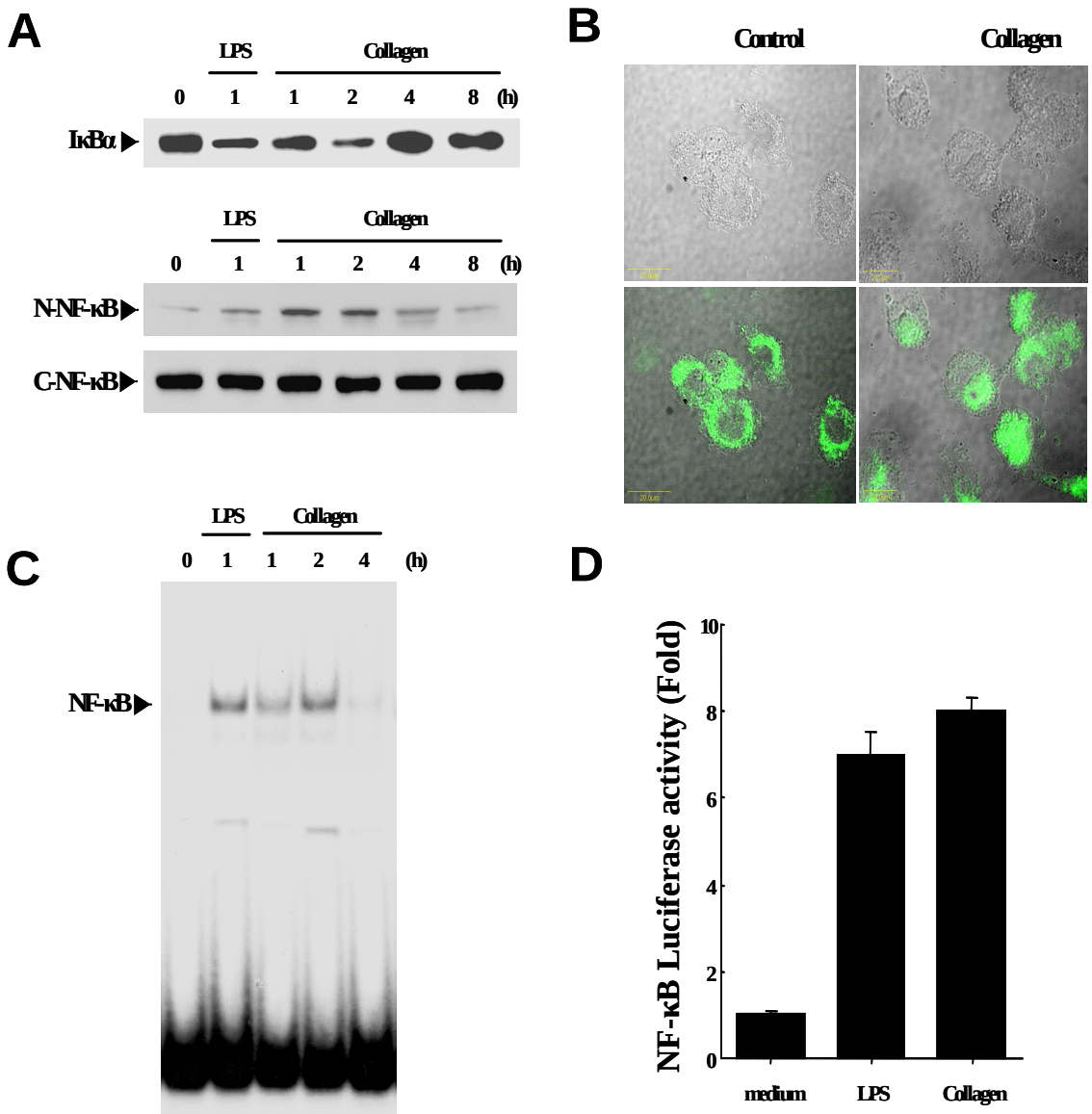

A
LPS
Collagen
0 1 1 2 4 8 (h)
IκBα
LPS
Collagen
0 1 1 2 4 8 (h)
N-NF-κB
C-NF-κB
B
Control
Collagen
C
LPS
Collagen
0 1 1 2 4 (h)
NF-κB
D
NF-κB Luciferase activity (Fold)
10
8
6
4
2
0
medium
LPS
Collagen

Figure 2. Collagen-induced IκBα degradation and NF-κB activation in J774 macrophages. A) Collagen-induced degradation of IκBα and translocation of NF-κB were measured by Western blot. Cells (1×10^6 cells/ml) were incubated with collagen (50 μg/ml) or LPS (30 ng/ml) at indicated times. B) Nuclear translocation of NF-κB was also evaluated by immunofluorescent staining using anti-p65 NF-κB antibody and FITC-labeled secondary antibody after 2 h of collagen (50 μg/ml) treatment. C) Cells were incubated with collagen (50 μg/ml) or LPS (30 ng/ml) at indicated times. Nuclear extracts were prepared and the p65 NF-κB-DNA binding activities were evaluated by EMSA using a ^{32}P-labeled DNA probe corresponding to the binding site of NF-κB. D) Cells were transiently transfected with the NF-κB luciferase reporter construct or empty vector. Then the cells were incubated with collagen (50 μg/ml) or LPS (30 ng/ml) for 12 h. NF-κB-dependent transcription activity was determined by luciferase activity assay. These data are representative of three independent experiments. N-NF-κB: nuclear-NF-κB, C-NF-κB: cytoplasmic-NF-κB.

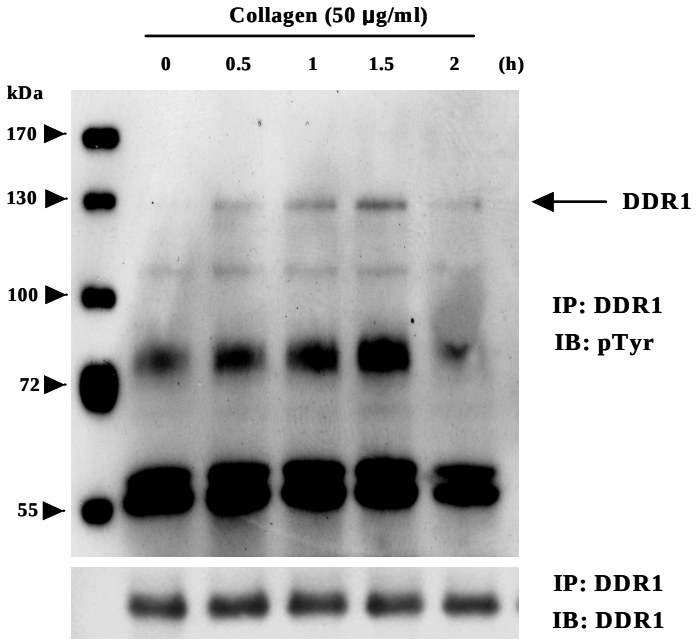


Figure 3. Expression and phosphorylation of DDR1 in J774 macrophages. Cells were incubated with collagen (50 μg/ml) for the indicated times. Ten million cells from each culture were lysed and the lysated were subjected to immunoprecipitation with anti-DDR1 antibody (C-20), and bound proteins were analyzed by Western blot.

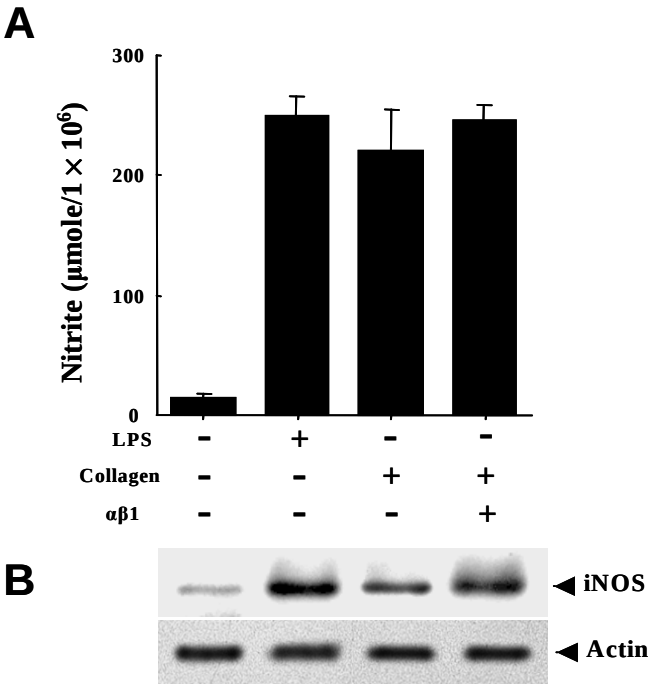


Figure 4. No alteration of collagen-induced iNOS and NO production by blocking β 1 integrin in J774 macrophages. Cells were pretreated with β1 integrin (10 μg/ml) blocking antibody for 30 min before collagen (50 μg/ml) or LPS (30 ng/ml) treatment. After 24 h of collagen or LPS stimulation, NO in the culture media was assayed by Griess reagent A), and iNOS protein production was assayed by Western blot B). Each bar represents the mean±SEM (n=3) of two independent experiments.

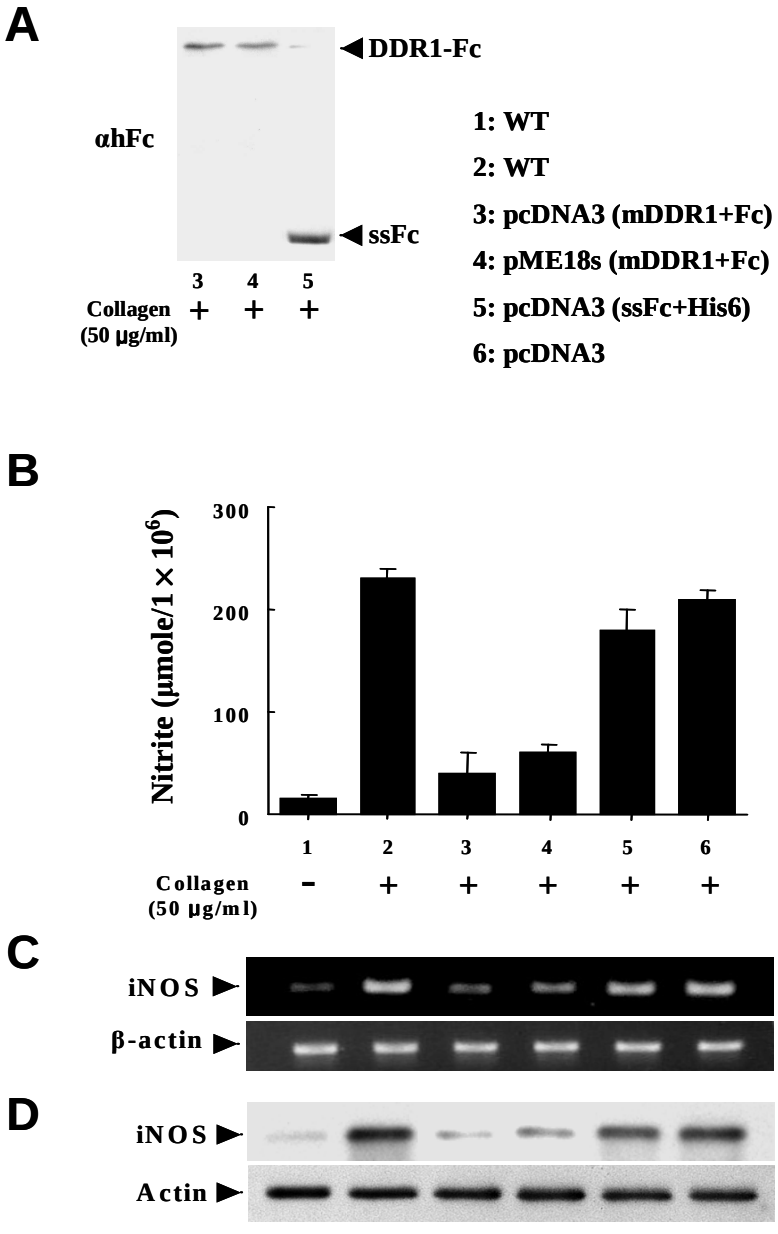

A
αhFc
DDR1-Fc
ssFc
3 4 5
Collagen + + +
(50 μg/ml)
1: WT
2: WT
3: pcDNA3 (mDDR1+Fc)
4: pME18s (mDDR1+Fc)
5: pcDNA3 (ssFc+His6)
6: pcDNA3
B
Nitrite (μmole/1 × 10^6)
300
200
100
0
1 2 3 4 5 6
Collagen - + + + + +
(50 μg/ml)
C
iNOS
β-actin
D
iNOS
Actin

Figure 5. Inhibition of iNOS and NO production with transfection of mouse DDR1 extracellular domain construct to J774 macrophages. Cells were transiently transfected with the mouse DDR1-Fc in pcDNA3, pME18s vector, ssFc-His6 in pcDNA3, and vector alone using Lipofectamine. A) After transfection, immunoblotting was performed using Ab against Fc. B) Eighteen hours after transfection, cells were stimulated with collagen (50 μg/ml) 24 h for NO production. C) Eighteen hours after transfection, cells were stimulated with collagen 6 h or 24 h for iNOS gene expression. D) Eighteen hours after transfection, cells were stimulated with collagen 6 h or 24 h for protein production, respectively. Each bar represents the mean±SEM (n=2) of two independent experiments.

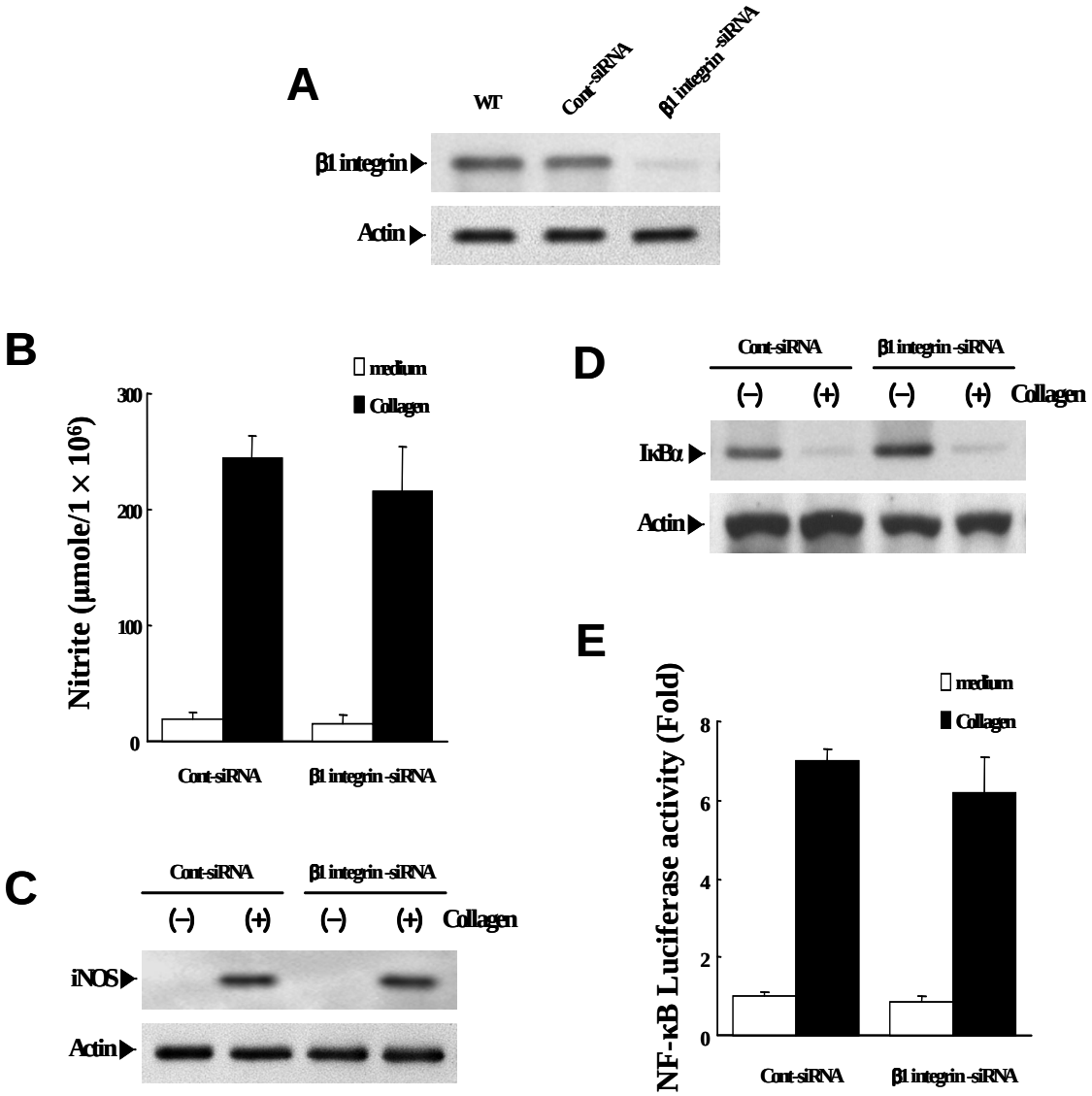
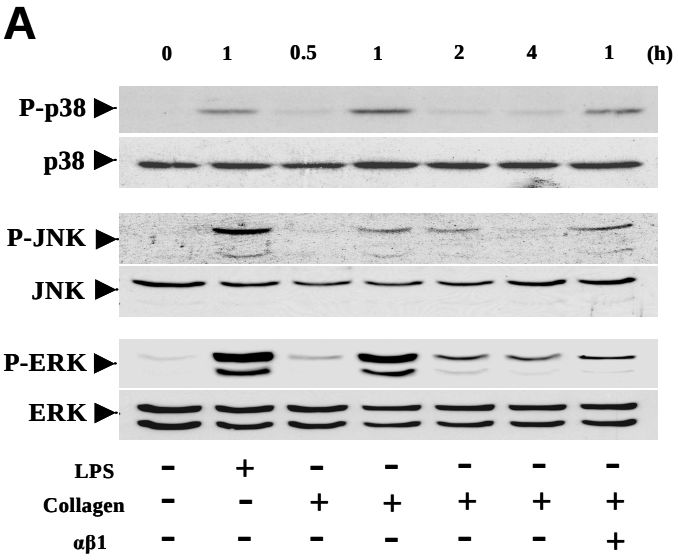

A
WT
Cont-siRNA
β1 integrin -siRNA
β1 integrin
Actin
B
medium
Collagen
Nitrite (μmole/1 × 10⁶)
300
200
100
0
Cont-siRNA
β1 integrin -siRNA
C
Cont-siRNA
β1 integrin -siRNA
(–) (+) (–) (+) Collagen
iNOS
Actin
D
Cont-siRNA
β1 integrin -siRNA
(–) (+) (–) (+) Collagen
IκBα
Actin
E
medium
Collagen
NF-κB Luciferase activity (Fold)
8
6
4
2
0
Cont-siRNA
β1 integrin -siRNA

Figure 6. No alteration of collagen-induced NF-κB activation in J774 macrophages transfected with β1 integrin-siRNA. A) Expression of β1 integrin in J774 cells was evaluated in control-siRNA or β1 integrin-siRNA treated cells by Western blot. Control-siRNA or β1 integrin-siRNA was transiently transfected to the cells. Then the cells were activated by collagen (50 μg/ml). Production of NO (B) and iNOS protein (C) were evaluated after 24 h of collagen stimulation. D) Degradation of Iκ Bα was measured after 2 h of collagen treatment. E) NF-κB-dependent transcription activity was determined by luciferase activity assay. These data are representative of two independent experiments.


A
0 1 0.5 1 2 4 1 (h)
P-p38
p38
P-JNK
JNK
P-ERK
ERK
LPS - + - - - - -
Collagen - - + + + + +
αβ1 - - - - - - +

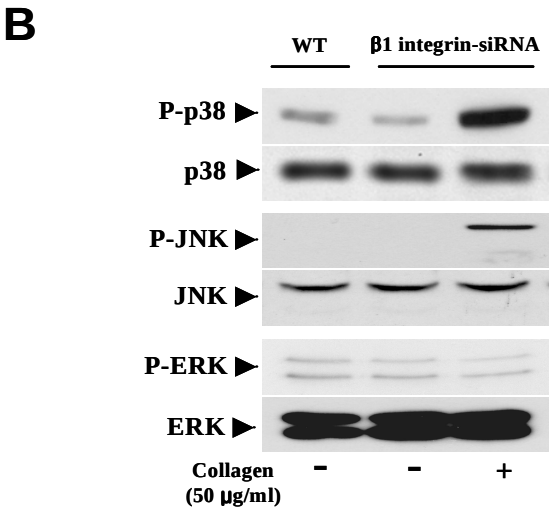

B
WT
β1 integrin-siRNA
P-p38
p38
P-JNK
JNK
P-ERK
ERK
Collagen (50 μg/ml) - - +

Figure 7. Differential role of three types of MAPKs on collagen-induced DDR1 signaling in J774 macrophages. A) Cells were incubated with collagen (50 μg/ml) or LPS (30 ng/ml) at indicated times. Cells were pretreated with β1 integrin blocking antibody (10 μg/ml) for 30 min prior to collagen stimulation. The phosphorylation of p38, JNK, and ERK were evaluated by Western blot. B) β1 integrin-siRNA transfected or WT cells were activated by collagen (50 μg/ml) for 1 h and phosphorylation of p38, JNK, and ERK were evaluated by Western blot.

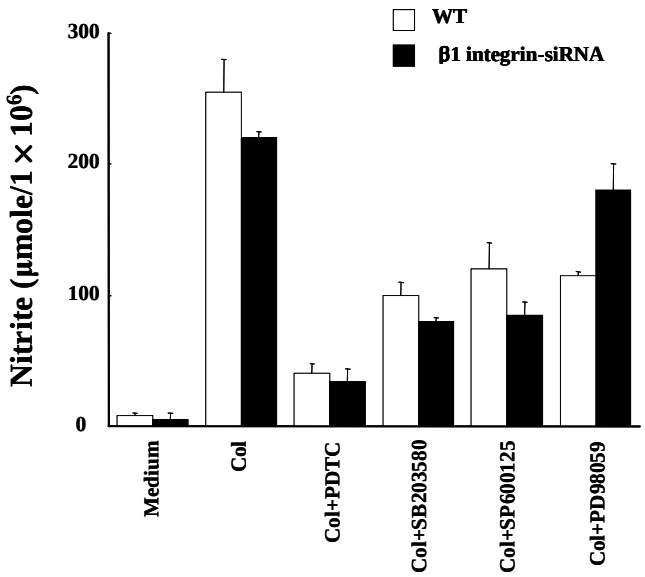


Figure 8. ERK phosphorylation is dependent on β1 integrin but not DDR1 signaling. β1 integrin-siRNA transfected or WT cells were activated by collagen for 24 h and production of NO in the culture media was assayed. PDTC (10 μM), SB203580 (5 μM), SP600125 (1 μM), and PD98059 (5 μM) were pretreated for 30 min prior to collagen (50 μg/ml) stimulation. Each bar represents the mean±SEM (n=3) of two independent experiments. Col: collagen

Ⅳ. DISCUSSION

It is well known that NO plays important roles in both physiological and pathophysiological cellular processes. Macrophages produce NO in response to proinflammatory stimuli and excess amounts of NO lead to increased production of inflammatory cytokines; thus, exerting proinflammatory process (Nussler and Billiar, 1993). Kim et al (Kim et al., 2002) previously reported that activation of a new class of collagen receptor, DDR1, facilitated the differentiation/maturation of human monocyte-derived macrophages and DCs and their cytokine/chemokine production via activation of p38 MAPK and NF-κB (Matsuyama et al., 2003a; Matsuyama et al., 2004). In the present study, I attempted to determine if activation of DDR1 with collagen also contributes to the expression of iNOS and subsequent NO production by macrophages using the mouse macrophage cell line J774. I have demonstrated that interaction of DDR1 with collagen up-regulates the expression of iNOS mRNA and protein, and concomitant production of NO dependently of p38 MAPK, JNK and NF-κB in those cells.

LPS is known to up-regulate the expression of various proinflammatory genes, including the iNOS gene, via toll-like receptor 4 (TLR4) (Akira., 2003). As presented above, the extent of iNOS expression in response to collagen was almost comparable to that in response to LPS. LPS and collagen had an additive effect, suggesting that collagen uses a receptor that is different from TRL4 to induced iNOS expression in macrophages. Recently, it was reported that type I collagen in combination with serum activated macrophages and stimulated NO production (Cho et al., 2002). The authors demonstrated that serum or collagen alone failed to induce iNOS gene expression, and suggested that the signaling pathway activated by collagen converged with that by serum for the induction of iNOS. Serum is known to activate various tyrosine kinases and the response of rat

mesangial cells to serum in combination with collagen was associated with the phosphorylation of tyrosine kinases (Zent et al., 1998). Based on these reports, I hypothesize that receptor tyrosine kinases could mediate collagen signaling for iNOS gene expression. Since there was no alteration in collagen-induced iNOS gene expression or NO production after blocking β1 integrins, it is likely that β1 integrins is not involved in the process.

I previously detected the induction of DDR1 in human leukocytes. Here, I have demonstrated that murine macrophage cell line J774 cells constitutively express DDR1. Stimulation of J774 cells with collagen resulted in tyrosine phosphorylation of DDR1. In addition, blocking β1 integrin with anti-β1 integrin blocking Ab had no effect on collagen-induced iNOS expression and NO production. These data suggested a role of DDR1 in collagen-induced iNOS expression and NO production.

It previously reported that NF-κB is a signaling molecule downstream of DDR1 using the human monocytic leukemic cell line THP-1 transduced to express DDR1 and monocyte-derived macrophages and DC that endogenously expressed DDR1 (Matsuyama et al., 2004). DDR1-mediated activation of NF-κB was regulated through the recruitment of the adaptor protein Shc, and a novel signaling cascade involving TRAF6 and Act1. NF-κB is a ubiquitous, pleotropic, multisubunit transcription factor activated in response to inflammatory and noninflammatory exogenous stimuli. In most cells, NF-κB is present as an inactive heterodimer, the predominant of which is composed of p50 and p65 subunits, and is sequestered within the cytoplasm by association with the inhibitory protein, IκB. Interaction of IκB with the NF-κB dimer prevents the nuclear translocation of NF-κB. Phosphorylation by IKKs leads to the ubiquitination and degradation of IκB, resulting in the nuclear translocation of NF-κB and subsequent activation of downstream target genes, including proinflammatory cytokines, chemokines and iNOS (Xie et al., 1994; Sha et al., 1995; Li and Verma, 2002). In the present

study, I examined the role of NF-κB in DDR1-mediated NO production. Stimulation of cells with collagen induced rapid degradation of IκB and nuclear translocation of NF-κB. Inhibition of β1 integrin expression by RNA interference did not alter collagen-induced degradation of IκB and nuclear translocation of NF-κB in macrophages. Thus, I have confirmed that NF-κB is a mediator of DDR1 signaling.

MAPKs are important mediators of cellular responses to extracellular signals. It previously reported the involvement of p38 MAPK in DDR1-mediated cytokine/chemokine productions, including IL-1β, IL-8, MIP-1α, and MCP-1, using human macrophages. p38 MAPK also mediated DDR1-mediated differentiation/maturation of human macrophages and DCs (Matsuyama et al., 2003b). There are several conflicted reports regarding the role of three types of MAPKs on iNOS gene expression. In case of costimulation with IFN-γ and TNF-α, all three types of MAPKs were activated. However, only JNK mediates IFN-γ and TNF-α mediated iNOS gene expression in mouse macrophages. In case of LPS stimulation, p38, but not ERK, mediates iNOS gene expression in RAW 264.7 macrophages (Chen and Wang, 1999). In the present study, I examined the role of all three types of MAPKs in DDR1-mediated NO production. Stimulation of J774 cells with collagen activated all three types of MAPKs. Interestingly, blocking of β1 integrin specifically reduced collagen-induced phosphorylation of ERK. These data suggest that p38 and JNK mediate DDR1 signaling, whereas ERK mediates β 1 integrin signaling in mouse macrophages. To confirm this hypothesis, we used siRNA to inhibit β1 integrin expression, and observed that ERK was not phosphorylated in those cells in response to collagen stimulation. In addition, specific inhibitor of p38 and JNK, but not ERK, decreased collagen-induced NO production in J774 cells. These data support our hypothesis that p38 and JNK mediate DDR1 signaling in iNOS gene induction. My results were consistent with the previous report showing that ERK was not a signaling molecule downstream of

DDR1 in human monocyte-derived macrophages (Matsuyama et al., 2003b).

Macrophages are one of the major cell types infiltrating the sites of chronic inflammatory diseases, such as atherosclerosis, pulmonary fibrosis, and rheumatoidarthritis. They release an array of cytokines, chemokines and NO, and contribute to the development of these diseases. My data presented here have indicated that DDR1-collagen interaction induces macrophage expression of iNOS and concomitant NO production, providing a novel mechanism by which macrophages produce NO in a tissue microenvironment in the course of inflammatory responses. Therefore, intervention of DDR1-collagen interaction or DDR1 signaling may be useful to control the development of inflammatory diseases in which NO plays an important role.

Ⅴ. SUMMARY & CONCLUSION

Nitric oxide (NO) is an important regulator of immune responses. Among different cell types, macrophages are the major producers of NO. Effects of cytokines, such as tumor necrosis factor-α or interferon-ɣ, and bacterial products, such as lipopolysaccharide, on macrophage NO production have been well documented; however, the role of the extracellular matrix proteins, such as collagen, in this process remains unclear.

Results can be summarized as follows

1. The activation of DDR1 with collagen induces the expression of inducible NO synthase (iNOS) mRNA and subsequent NO production in mouse macrophages.
2. Neither β1 integrin blocking antibody nor β1 integrin siRNA inhibited collagen-induced iNOS expression, indicating that it was not due to collagen-β1 integrin interaction.
3. Nuclear factor-κB, p38 and c-jun N-terminal kinase mitogen-activated protein kinase were crucial mediators of collagen-induced downstream DDR1 signaling in macrophages, whereas extracellular signal-regulated kinase was not.

Thus, this study demonstrates that collagen activation of DDR1 up-regulates NO synthesis by macrophages and suggests that intervention of DDR1 signaling may be useful to control inflammatory diseases in which NO plays a critical role.

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Ⅶ. REFERENCES

Akira, S.. "Toll-like receptor signaling." *J Biol Chem.* **278**:38105-38108, 2003.

Bhatt, R.S. Tomoda, T. Fang, Y. and Hatten, M.E.. "Discoidin domain receptor 1 functions in axon extension of cerebellar granule neurons." *Genes Dev.* **14**:2216-2228, 2000.

Chan, E.D. Winston, B.W. Uh, S.T. Wynes, M.W. Rose, D.M. and Riches, D.W.. "Evaluation of the role of mitogen-activated protein kinases in the expression of inducible nitric oxide synthase by IFN-gamma and TNF-alpha in mouse macrophages." *J Immunol.* **162**:415-422, 1999.

Chen, C.C. and Wang, J.K.. "p38 but not p44/42 mitogen-activated protein kinase is required for nitric oxide synthase induction mediated by lipopolysaccharide in RAW 264.7 macrophages." *Mol Pharmacol.* **55**:481-488, 1999.

Cho, M.K. Suh, S.H. and Kim, S.G.. "JunB/AP-1 and NF-kappa B-mediated induction of nitric oxide synthase by bovine type I collagen in serum-stimulated murine macrophages." *Nitric Oxide* **6**:319-332, 2002.

Gross, S.S. and Wolin, M.S.. "Nitric oxide: pathophysiological mechanisms." *Annu. Rev Physiol.* **57**:737-769, 1995.

Hou, G. Vogel, W. and Bendeck, MP.. "The discoidin domain receptor tyrosine kinase DDR1 in arterial wound repair." *J Clin Invest.* **107**:727-735, 2001.

Hou, G. Vogel, W.F. and Bendeck, M.P.. "Tyrosine kinase activity of discoidin domain receptor 1 is necessary for smooth muscle cell migration and matrix metalloproteinase expression." *Circ Res.* **90**:1147-1149, 2002.

Jonsson, M. and Andersson, T.. "Repression of Wnt-5a impairs DDR1

phosphorylation and modifies adhesion and migration of mammary cells." *J Cell Sci.* **114**:2043-2053, 2001.

Kim, S.H. Johnson, V.J. and Sharma, R.P.. "Mercury inhibits nitric oxide production but activates proinflammatory cytokine expression in murine macrophage: differential modulation of NF-kappaB and p38 MAPK signaling pathways." *Nitric Oxide*. **7**:67-74, 2002.

Kim, S.H. Johnson, V.J. Shin, T.Y. and Sharma, R.P.. "Selenium attenuates lipopolysaccharide-induced oxidative stress responses through modulation of p38 MAPK and NF-kappaB signaling pathways." *Exp Biol Med (Maywood)*. **229**:203-213, 2004.

Li, Q. and Verma, I.M.. "NF-kappaB regulation in the immune system." *Nat Rev Immunol.* **2**:725-734, 2002.

MacMicking, J. Xie, Q.W. and Nathan, C.. "Nitric oxide and macrophage function." *Annu Rev Immunol.* **15**:323-350, 1997.

Matsuyama, W. Faure, M. and Yoshimura, T.. "Activation of discoidin domain receptor 1 facilitates the maturation of human monocyte-derived dendritic cells through the TNF receptor associated factor 6/TGF-beta-activated protein kinase 1 binding protein 1 beta/p38 alpha mitogen-activated protein kinase signaling cascade." *J Immunol.* **171**:3520-3532, 2003a.

Matsuyama, W. Kamohara, H. Galligan, C. Faure, M. and Yoshimura, T.. "Interaction of discoidin domain receptor 1 isoform b (DDR1b) with collagen activates p38 mitogen-activated protein kinase and promotes differentiation of macrophages." *Faseb J.* **17**:1286-1288, 2003b.

Matsuyama, W. Wang, L. Farrar, W.L. Faure, M. and Yoshimura, T.. "Activation of discoidin domain receptor 1 isoform b with collagen up-regulates chemokine production in human macrophages: role of p38 mitogen-activated protein kinase and NF-kappa B." *J Immunol.* **172**:2332-2340, 2004.

May, M.J. and Ghosh, S.. "Signal transduction through NF-kappa B." *Immunol Today.* **19**:80-88, 1998.

Nussler, A.K. and Billiar, T.R.. "Inflammation, immunoregulation, and inducible nitric oxide synthase." *J Leukoc Biol.* **54**:171-178, 1993.

Ongusaha, P.P. Kim, J.I. Fang, L. Wong, T.W. Yancopoulos, G.D. Aaronson, S.A. and Lee, S.W.. "p53 induction and activation of DDR1 kinase counteract p53-mediated apoptosis and influence p53 regulation through a positive feedback loop." *Embo J.* **22**:1289-1301. 2003

Pacifici, R. Carano, A. Santoro, S.A. Rifas, L. Jeffrey, J.J. Malone, J.D. McCracken, R. and Avioli, L.V.. "Bone matrix constituents stimulate interleukin-1 release from human blood mononuclear cells." *J Clin Invest.* **87**:221-228, 1991.

Seger, R. and Krebs, E.G.. "The MAPK signaling cascade." Faseb J **9**:726-735. 1995

Sha, W.C. Liou, H.C. Tuomanen, E.I. and Baltimore, D.. "Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses." *Cell.* **80**:321-330, 1995.

Vogel, W.. "Discoidin domain receptors: structural relations and functional implications." *Faseb J.* **13 Suppl**:S77-82, 1999.

Vogel, W. Brakebusch, C. Fassler, R. Alves, F., Ruggiero, F. and Pawson, T.. "Discoidin domain receptor 1 is activated independently of beta(1) integrin." *J Biol Chem.* **275**:5779-5784, 2000.

Vogel, W. Gish, G.D. Alves, F. and Pawson, T.. "The discoidin domain receptor tyrosine kinases are activated by collagen." *Mol Cell.* **1**:13-23, 1997.

Xie, Q.W. Kashiwabara, Y. and Nathan, C.. "Role of transcription factor NF-kappa

B/Rel in induction of nitric oxide synthase." *J Biol Chem.* **269**:4705-4708, 1994.

Zent, R. Ailenberg, M. and Silverman, M.. "Tyrosine kinase cell signaling pathways of rat mesangial cells in 3-dimensional cultures: response to fetal bovine serum and platelet-derived growth factor-B." *Exp Cell Res.* **240**:134-143, 1998.