Effect of nitric oxide on the expression of matrix metalloproteinases by the UV irradiated human dermal fibroblasts

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The production of matrix matalloproteinases(MMPs) by the UV irradiated skin fibroblast and the degradation of extracellular matrix(ECM) by these enzymes is known as one of the main reasons of photoaging. Recently, Fisher group showed that the MMP expression is mainly regulated by the mitogen-activated protein(MAP) kinas family, such as extracellular signal-regulated kinase(ERK), c-Jun amino-terminal kinase(JNK) and p38, each of which forms a signaling pathway. In this work, we first examined the effect of nitric oxide (NO) on the production of MMP-1 and MMP-2 by the human dermal fibroblasts (HDFs). NO is a multifunctional messenger molecule generated from L-arginine and involved in many kinds of signaling pathway. We found that the treatment of HDF with NO donor, sodium nitroprusside (SNP) enhanced the expression of MMPs with or without UV irradiation and the treatment with nitric oxide synthase (NOS) inhibitors resulted in the significant decrease of MMPs production. From these results, we concluded that the production of MMPs by the UV irradiated HDF is regulated through the signaling pathway involving NO and cyclic GMP.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of enzymes that are responsible to the degradation of extracellular matrix (ECM) components such as collagen, laminin and proteoglycans. Among the MMPs, MMP-1 is interstitial collagenase that degrades fibrillar collagens and proteogycans and

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MMP-2 is gelatinase that degrades denatured collagens and elastin. The main reason for the connective tissue changes in the UV irradiated skin has been clarified as the production of MMPs including MMP1 and MMP2 (1-5) and the degradation of ECM components by these enzymes. The expression of MMPs in UV irradiated fibroblasts is known to be initiated by the singlet oxygen (6), alpha-melanocyte stimulating hormone produced by keratinocytes(7) or by the activation of cell surface growth factor and cytokine receptor, which mimics the actions of receptor ligands (8, 9). Recently, Fisher et al. (10) showed that the MMP expression is mainly regulated by the mitogen-activated protein(MAP) kinas family, such as extracellular signal-regulated kinase(ERK), c-Jun amino-terminal kinase(JNK) and p38, each of which forms a signaling pathway. In some types of cells, the expression of MMPs is mediated by nitric oxide (NO) (11, 12). NO is a multifunctional messenger molecule generated from L-arginine by a family of enzymes, including nitric oxide synthase(NOS). The production and diffusion of NO triggering the melanogenesis of melanocytes by the UV irradiated keratinocytes is well documented (13), but relatively little is known about the effect of NO on the production of MMPs by the epidermal fibroblasts, which are also under the influences of cytokines released from keratinocytes. In this work, we first examined the effect of NO on the production of MMP-1 and MMP-2 by the human dermal fibroblasts (HDFs). We found that the treatment of HDF with NO donor, sodium nitroprusside (SNP) enhanced the expression of MMPs with or without UV irradiation and the treatment with nitric oxide synthase (NOS) inhibitors resulted in the significant decrease of MMPs production. From these results, we concluded that the production of MMPs by the UV irradiated HDF is also regulated through the signaling pathway involving NO and cyclic GMP.

MATERIALS AND METHODS

Reagents

Sodium Nitroprusside(SNP) (14,15), Aminoguanidine , Anti-mouse IgG for the secondary antibody, lipopolysaccharide(LPS) was purchased from Sigma chemical Co. Anti-MMP-1 antibody (Ab-5), anti-MMP-2 antibody(Ab-3) and anti- mouse IgG conjugated with alkaline phosphatase were obtained from Cal- biochem.

Culture of Human dermal fibroblast

HDFs from new born foreskin were acquired from Korea Cancer Center Hospital. HDFs were

maintained in Dulbecco's Modified Eagle's Media(DMEM) with 10% FBS and kept in a humidified 5% CO_2 atmosphere at 37℃. HDFs from passage 6 to 10 were used in the experiments.

UV irradiation

HDFs(1.5×10^5/well) were seeded into 35ø plates and cultured overnight. Prior to UV irradiation, the cells were washed twice with phosphate buffered saline(PBS). The cells were irradiated from a distance of 15 cm by a UV source emitting wavelengths in the range of 340 - 450 nm. The culture media, DMEM containing no serum, was added and incubated for 12 hours. The concentration of MMP1 and 2 in the culture media was determined as described below.

Determination of MMP 1 and 2 by ELISA

The expression of MMP 1 and 2 was assayed by enzyme-linked immunosorbent assay(ELISA). HDFs(8×10^3/well) were seeded into 96-well plates and cultured overnight. The culture media were replaced with DMEM containing 10% FBS, SNP and/or AG. After 12 hours' incubation, the supernatants were transferred into a 96 well plate and the coating buffer (Na_2CO_3 1.59%, $NaHCO_3$ 2.93%, NaN_3 0.20%, $MgCl_2$ 1.02%, pH 9.6) was added 1:1(v/v) and incubated for 12 hours. The supernatants were removed and the coated well was washed with PBS-T for 3 times and followed by blocking with 5% skim milk in PBS for 1 hour at 37℃. After washing 3 times with PBS-T, 50㎕ of 1/1000 diluted primary antibody(Ab), Ab-5 or Ab-3 in PBS-T were added into each well and incubated for 40 min. Washing the wells with PBS-T 3 times, 50㎕ of 1/1000 diluted secondary Ab, anti-mouse IgG conjugated with alkaline phosphatase in PBS-T was added and incubated for 40 min. After 5 times washing with PBS-T, 100㎕ of 1mg/ml pNPP(p-nitrophenyl phosphate) in diethanolamine buffer was added. The optical density was measured at 405nm after 30 min. Cytotoxicity of supplemented chemicals was measured by 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

RT-PCR-ELISA

Total RNA was extracted from cultured cells using the Promega RNAgent® Total RNA isolation System. 1㎍ of extracted RNA was reverse-transcribed and amplified using an Access RT-PCR system Kit (Promega) on a thermal cycler(Perkin Elmer GeneAmp. PCR system 2400). First-strand cDNA was synthesized by reverse transcription at 48℃ for 45 minutes in a 50 ㎕ reaction mixture ,

and then, heating at 94℃ for 2 minutes for denaturing. PCR was performed on a thermal cycler using a program of 28 cycles(MMP-1), 27 cycles(MMP-2) at 94℃ for 30 minutes. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH, 26 cycles) was used as internal control. Specific primers were as follows (15):

MMP-1(sense) 5'-GGA-GAT-CAT-CGG-GAC-AAC-T-3'
MMP-1(anti-sense) 5'-GGG-TAT-CCG-TGT-AGC-ACA-TTC-3'
MMP-2(sense) 5'-TGG-GAG-CAT-GGC-GAT-GGA-TA-3'
MMP-2(anti-sense) 5'ACA-GTG-GAC-ATG-GCG-GTC-TCA-3'
Human iNOS(sense) 5'-AGTTTCTGGCAGCAACGG -3'
Human iNOS(anti-sense) 5'-TTAAGTTCTGTGCCGGCAG -3'

After a given cycle of PCR, the amount of amplified cDNA was determined by the PCR-ELISA method developed in our laboratory.

RESULTS AND DISCUSSION

Effect of UV on the production of MMPs

The immunoreactive MMP-1 and 2 in the culture medium of HDFs were measured using anti MMP-1 and 2 monoclonal antibodies, respectively. Treatment of HDFs with UV radiation enhanced the production of MMP-1 by 2-fold and MMP-2 by 3-fold in a dose-related manner (Fig 1), confirming the previous results (1-5). We also confirmed that the gelatinase activities were proportionally increased with the UV irradiation of HDFs using gelatin zymography. (Data not shown) The production of MMPs by UV irradiation of HDFs is a result of the activation of cell surface growth factors and cytokine receptors, which have a common the requirement for dimerization to initiate signal transduction. UV radiation rapidly activates EGF receptors following by the activation of Ras, ERK, JNK and p38(17, 18). These stress-activated MAP kinases then increase the proteins of c-Jun, c-Fos and ATF. The dimerization of these proteins activate AP-1 DNA binding, finally the induction of MMPs. Fisher et al. showed that retinoic acid can inhibit the induction of MMPs by UV irradiation in human skin by blocking the DNA binding of AP-1 and c-Jun protein induction (19).

Effect of nitric oxide donor on the production of MMPs

To determine the effect of NO on the production MMP-1 and 2, HDFs were treated with SNP. SNP is a donor of NO and it can mimic the cellular effects of NO. Treatment with SNP increased MMP-2 production acutely, about 243% of untreated cells with 50 microM of SNP. The amount of secreted MMP-1 in the HDF culture medium was also increased with SNP treatment, but less significant than that of MMP-2, about 153%. (Fig.2A). The combined effect of SNP and UV treatment was also tested as shown Fig. 2B. The null hypothesis was that the effect of UV irradiation is not mediated by NO, subsequently, the addition of SNP to UV irradiated HDF should increase MMP production to the same degree of as in cells not treated with UV irradiation and more than in cells treated only with UV irradiation. In the UV treated cells, 50 microM SNP didn't increase MMP1 production significantly, while that of MMP2 increased by 196%. (Fig. 2B) This result indicates that the effect of UV irradiation and SNP treatment on the MMP1 production by HDF does not summate, which means that the UV irradiation already produced a near-maximum increase in the production of MMP1 and the addition of 50 microM SNP could not increase the MMP1 production further. On the other hand, the production of MMP2 seems to be influenced by both the UV irradiation and SNP treatment at the same time.

Effect of UV on the production of iNOS

The production of nitric oxide in UV-irradiated keratinocytes was reported by Romero-Graillet et al. (21) and they confirmed that NO played an important role in the paracrine mediation of UV-induced melanogenesis. Fig. 3 shows the expression of inducible NOS (iNOS) and the production of NO from HDF treated with UV radiation of 30J/cm^2. The mRNA of iNOS was increased by 12% when HDF was treated with UV of 30J/cm^2. The amount of NO produced by UV irradiated HDF is not so significant, however, if we also count the NO produced by the UV irradiated keratinocytes *in vivo* system, the influence of NO on the fibroblast should not be negligible. LPS, a microbial cell wall component, is a good stimulator for NO production by immune cells, including macrophages. This is the first time that LPS was used as the stimulator of NO production in HDF, not an immune cell, and there was a significant increase(42%) in iNOS in LPS treated HDF.(Fig. 3)

Inhibition of MMP production by iNOS

To better understand the mechanism of action of SNP, two experiments were performed. In the first

experiment, HDFs were treated with AG and SNP or AG and LPS and then the secreted proteins or the intracellular mRNA of MMP-1 or MMP-2 were determined. AG is a NOS inhibitor that inhibits both the constitutive and inducible NOS. The enhancement of MMP production by SNP was partially but significantly blocked by an NO synthase inhibitor, AG. Fig. 4A shows that the enhanced production of MMP-1 or MMP-2 by SNP or LPS was reversed by the addition of AG. When administered alone, AG, it had an effect on the deregulation of MMP production. The results were confirmed again by determining the mRNA of MMPs. (Fig. 4B) These findings together suggests that NO affects on the MMP production through the c-GMP pathway but this effect is not addible to the effect of UV radiation or the role of NO is . partially regulated by signaling pathway involved NO.

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Fig.1 MMP1 and 2 production in the UV irradiated HDF

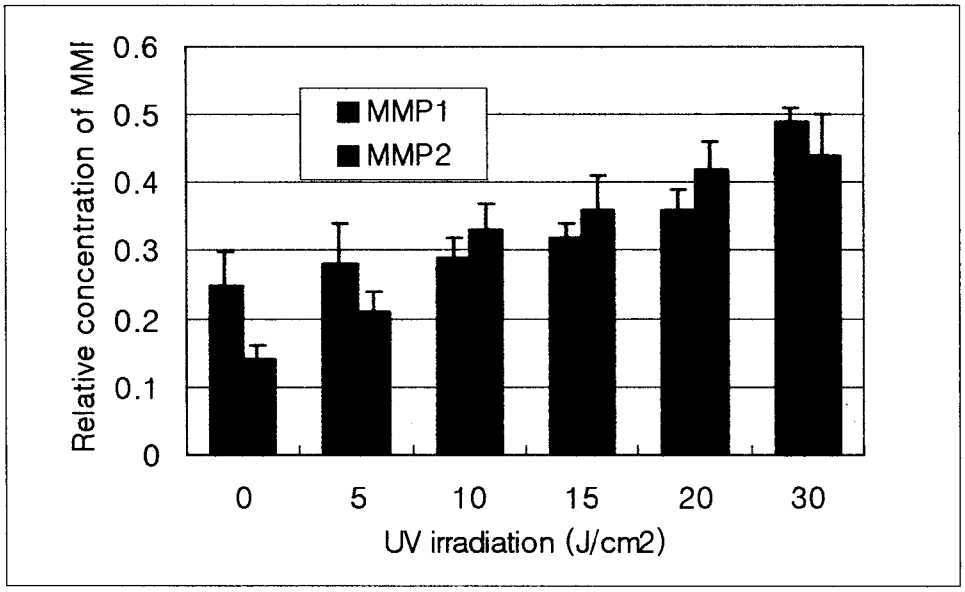


Fig. 2A MMP 1 and 2 production by NO donors;SNP (without UV irradiation)

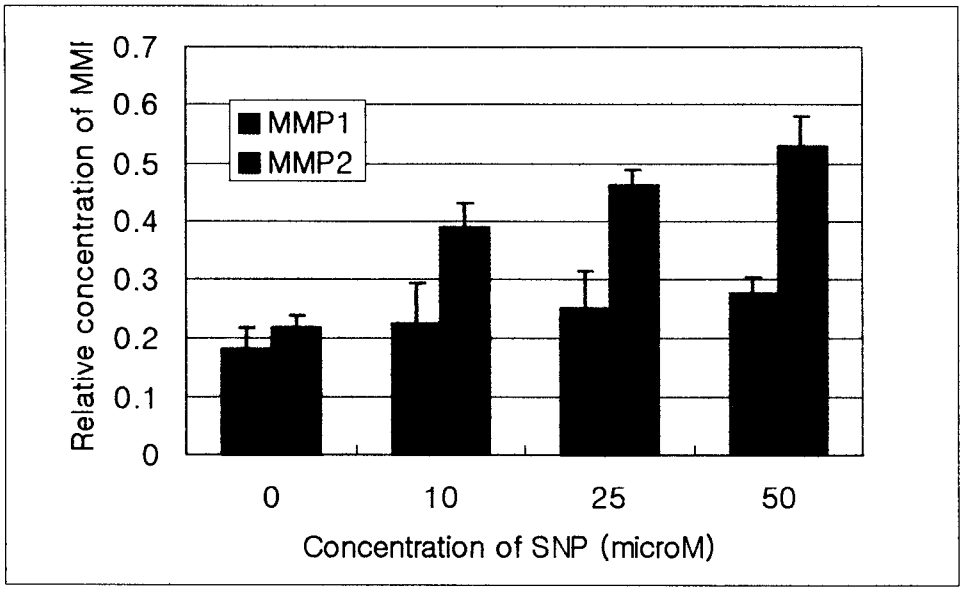


Fig. 2B MMP 1 and 2 production by NO donors

(with UV irradiation)

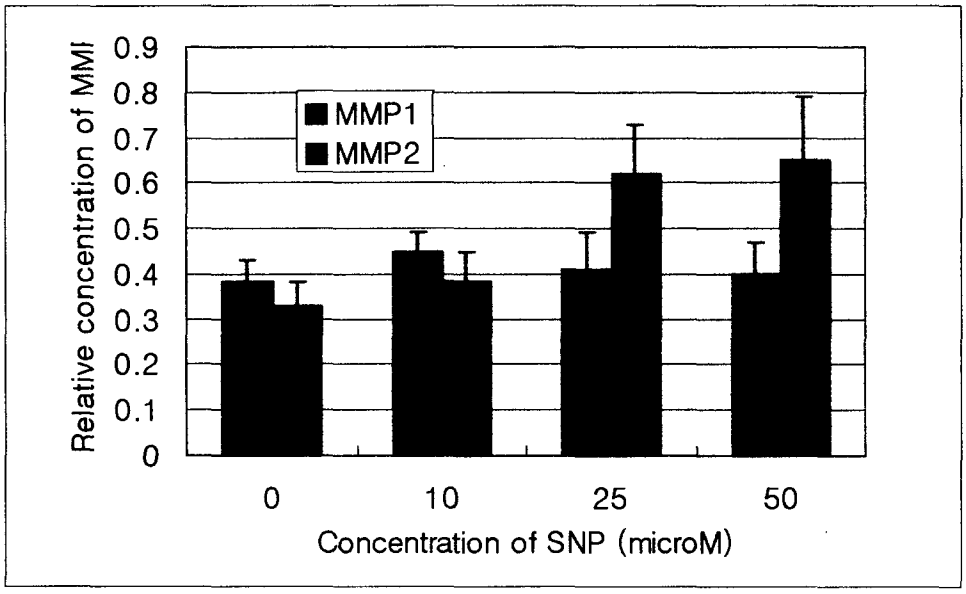


Fig. 3 Inducible nitric oxide synthase(iNOS) production by UV irradiation

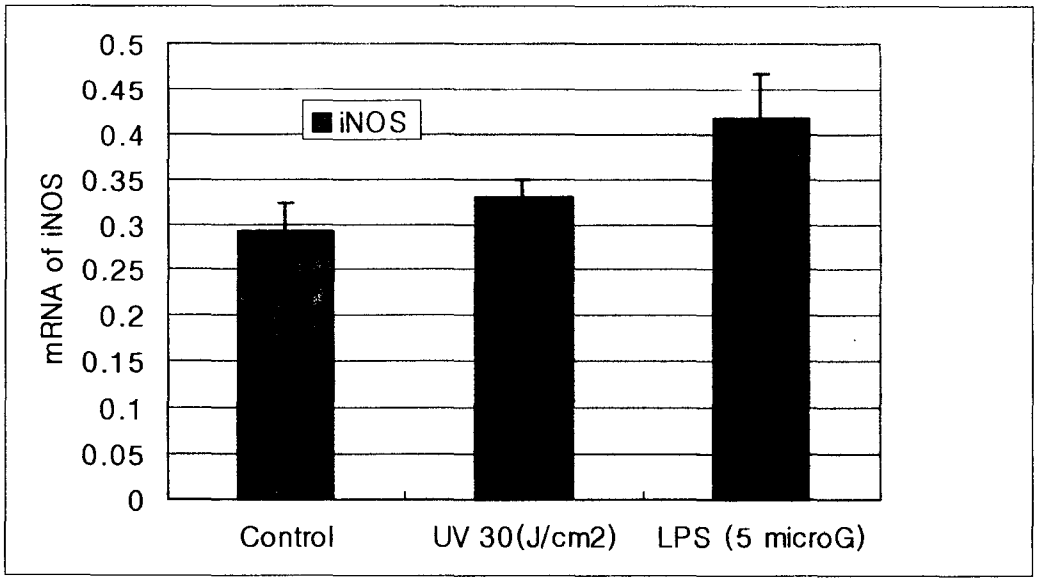


Fig. 4A Inhibition of MMP production by iNOS inhibitor;AG (protein)

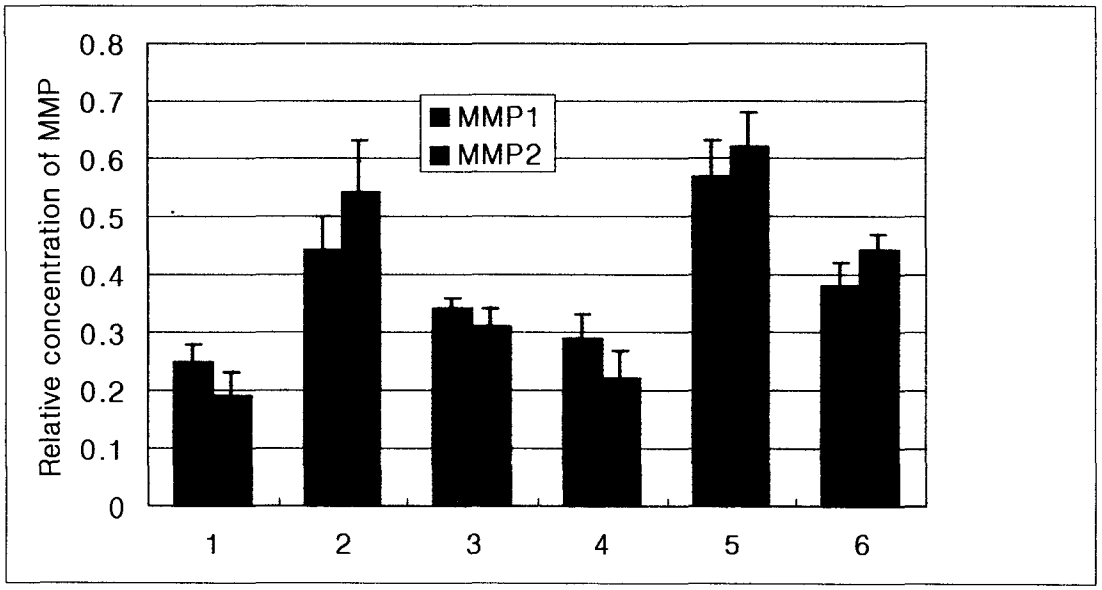
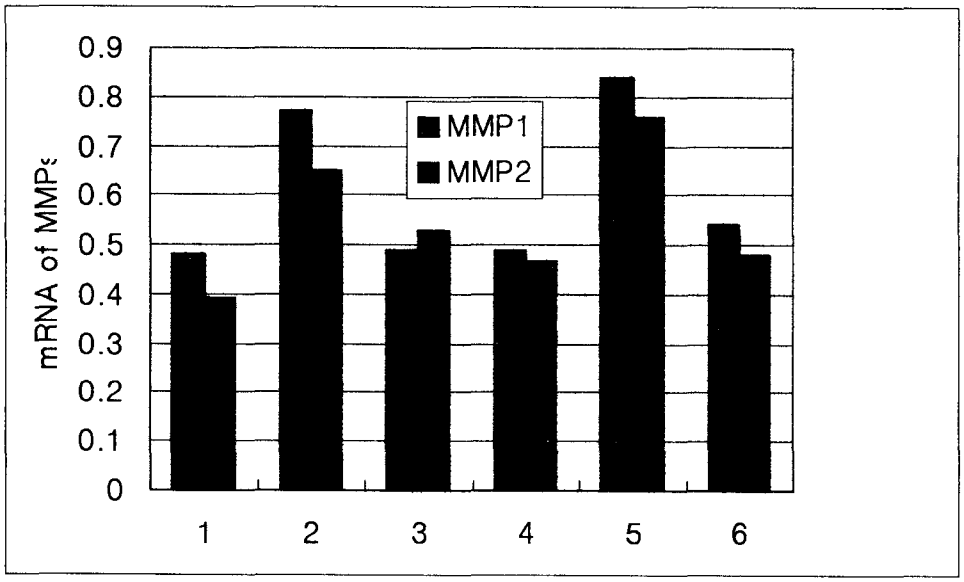


Fig. 4B Inhibition of MMP production by iNOS inhibitor;AG (mRNA)



1. Control 2. SNP (50microM) 3. SNP + AG (25microM)
4. SNP + AG (50microM) 5. LPS (5 microgram) 6. LPS + AG(50microM)