# Cyclooxygenase-2 Expression and Prostaglandin $E_2$ Biosynthesis Are Enhanced in Scleroderma Fibroblasts and Inhibited by UVA Irradiation

TAKURO KANEKURA, YUKO HIGASHI, and TAMOTSU KANZAKI

***ABSTRACT.*** ***Objective.*** We and others reported on the beneficial effects of combined therapy using 8-methoxypsoralen and long wave ultraviolet light (PUVA therapy) in the treatment of scleroderma. We now investigate the mechanism by which PUVA therapy is effective by comparing interleukin 1ß (IL-1ß) mediated signal transduction in scleroderma fibroblasts and those from normal skin.

***Methods.*** Prostaglandin $E_2$ ($PGE_2$) production and expression of cytosolic phospholipase $A_2$ ($cPLA_2$), cyclooxygenase (COX)-1, and COX-2 (enzymes that regulate $PGE_2$ production) were examined in untreated and IL-1ß treated fibroblasts from scleroderma involved and normal skin. The effect of UVA irradiation on enzyme expression and $PGE_2$ production was examined. $PGE_2$ was measured by a competitive radioimmunoassay and enzyme expression was analyzed by Western immunoblotting and Northern blotting.

***Results.*** Constitutive $PGE_2$ production was significantly upregulated and IL-1ß induced $PGE_2$ production was increased by the enhancing expression of both COX-2 mRNA and protein in fibroblasts from scleroderma involved skin; $PGE_2$ production and COX-2 expression were inhibited by UVA irradiation.

***Conclusion.*** Enhanced $PGE_2$ production regulated by COX-2 expression in scleroderma fibroblasts may contribute to the development of this disorder. PUVA therapy might exhibit its beneficial effect, at least in part, by inhibiting COX-2 expression transcriptionally and translationally, with subsequent inhibition of $PGE_2$ production. (J Rheumatol 2001;28:1568–72)

*Key Indexing Terms:*
SCLERODERMA INTERLEUKIN 1ß CYCLOOXYGENASE-2
PROSTAGLANDIN $E_2$ UVA

Scleroderma is an inflammatory disorder of connective tissue that manifests itself as sclerosis of systemic organs, especially of the dermis; this is caused by increased synthesis of collagen by fibroblasts[1,2]. The underlying mechanism in the development of this disorder remains to be elucidated; studies show that interleukin 1 (IL-1) mediated signal transduction in dermal fibroblasts might be involved in the pathogenesis of scleroderma[3,4]. It was reported that fibroblasts produced prostaglandin $E_2$ ($PGE_2$) in response to IL-1ß treatment[5,6], and possible correlation between $PGE_2$ and collagen synthesis in scleroderma was discussed[7]. A variety of agents, including vitamin E, penicillamine, and cyclosporine, have been employed in the treatment of scleroderma, with only limited success. We and others reported successful treatment of scleroderma with combined therapy using 8-methoxypsoralen and long wave ultraviolet light (PUVA therapy)[8-10]. These facts led us to investigate the effects of long wave ultraviolet (UVA) irradiation on IL-1 mediated signal transduction in fibroblasts cultured from scleroderma and normal skin samples. We examined IL-1ß induced $PGE_2$ biosynthesis and the expression of cytosolic phospholipase $A_2$ ($cPLA_2$), cyclooxygenase-1 (COX-1), and COX-2, enzymes that regulate $PGE_2$ biosynthesis.

We show that constitutive and IL-1ß mediated $PGE_2$ production are increased in scleroderma fibroblasts by enhancing COX-2 expression, and that both COX-2 expression and $PGE_2$ production are clearly inhibited by UVA irradiation. These results suggest that altered IL-1ß related signal transduction may be involved in the pathogenesis of scleroderma, and that the possible reason PUVA therapy is beneficial in treating scleroderma is that it inhibits COX-2 expression.

## MATERIALS AND METHODS

*Patients and samples.* Five female patients, ages 52, 63, 80, 43, and 65 years, were diagnosed with scleroderma based on clinical and histopathological findings. Before patients started treatments, skin specimens were obtained from their involved middle fingers and uninvolved abdomens. Abdominal skin samples were obtained from 4 healthy control subjects.

*From the Department of Dermatology, Faculty of Medicine, Kagoshima University, Kagoshima, Japan.*

*Supported in part by grant no. 11670840 from the Ministry of Education, Science and Culture of Japan.*

*T. Kanekura, MD, PhD, Associate Professor; Y. Higashi, MD, PhD, Resident; T. Kanzaki, MD, PhD, Professor and Chairman.*

*Address reprint requests to Dr. T. Kanekura, Department of Dermatology, Kagoshima University Faculty of Medicine, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan. E-mail: takuro1@m2.kufm.kagoshima-u.ac.jp*

*Submitted June 9, 2000; revision accepted January 2, 2001.*

*Cell culture*. Dermal fibroblasts were cultured as described[11]. Skin specimens were minced and placed in sterile 3 cm dishes. The tissue was pressed by a cover slip and incubated with 3 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 100,000 units/l penicillin G, 100 mg/l streptomycin, 1 mg/l Fungizone, 0.1 mM nonessential amino acids, 292 mg/l glutamine, 50 mg/l ascorbic acid, and 10% fetal calf serum under sterile conditions at 37°C in a humidified atmosphere containing 5% $CO_2$. The media were changed every 3 days until cells became confluent (4–6 wks), then cells were seeded at $10^5$ cells/ml in identical medium in 24 well flat bottom tissue culture plates for $PGE_2$ measurement and in 10 cm tissue culture dishes for protein extraction.

***UVA source and irradiation*.** T-15L black light tubes (ATTO, Tokyo, Japan) emitting radiation at 365 nm were used as the UVA source. UVA irradiation was performed through the bottom of plates. UVA intensity was measured at 365 nm with a UV radiometer (UVR-305/365, Eisai, Tokyo) and was 5 mW/cm$^2$.

***$PGE_2$ measurement*.** $PGE_2$ was measured by radioimmunoassay[12]. The method is based on the competition of $PGE_2$ in the test sample with [$^3$H]-labeled $PGE_2$ (DuPont-NEN, Boston, MA, USA) for binding to anti-$PGE_2$ antibody (Upstate Biotechnology, Lake Placid, NY, USA). Cells were seeded at $1 \times 10^5$ cells/ml in 24 well tissue culture plates (0.5 ml/well) and incubated 72 h until confluent. The media were then replaced with fresh DMEM containing 1% fetal calf serum and IL-1ß at appropriate concentrations. A 10 to 100 $\mu$l aliquot of culture medium was added to radioimmunoassay buffer (0.1 mM phosphate buffer, pH 7.4, containing 0.9% sodium chloride, 0.1% sodium azide, and 0.1% gelatin), mixed with the appropriate amounts of labeled $PGE_2$ and reconstituted antiserum, and incubated overnight at 4°C. The assay tubes were then placed on ice, and 1 ml of cold charcoal-dextran suspension was added. After 15 min, the tubes were centrifuged at 2200 × g for 10 min at 4°C. The supernatants were decanted into scintillation vials and radioactivity was determined by scintillation spectrometry. The percentage of binding was compared to a standard curve and the amounts of $PGE_2$ in the samples were calculated.

*Western immunoblotting*. Fibroblasts from scleroderma and uninvolved skin were seeded at $1 \times 10^5$ cells/ml in 10 cm tissue culture plates (10 ml/plate) and incubated until they were confluent. Fibroblasts were pelleted in PBS containing 1 $\mu$g/ml leupeptin, 0.03% Aprotinin, 2 mM PMSF, and 2 mM EDTA, pH 7.2. Proteins were extracted by suspending the pellet in extraction buffer (62.5 mM Tris, 2% SDS, 0.5 mM DTT, 1 mM fresh PMSF, pH 6.8) and denaturing the proteins at 95°C for 5 min. Identical amounts (20 $\mu$g) of protein were electrophoresed in a 7.5% SDS/polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 and incubated with either anti-COX-1, anti-COX-2, or anti-$cPLA_2$ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. After incubation with anti-rabbit alkaline phosphatase linked immunoglobulin for 1 h at room temperature, the immunoreactive bands were detected using an ECL kit (Amersham, Arlington Heights, IL, USA).

*Northern blotting*. Total RNA was extracted from fibroblasts from scleroderma and uninvolved skin grown in 10 cm dishes using the TRIzol reagent (Life Technologies Inc., Frederick, MD, USA). Twenty micrograms of total RNA was denatured at 50°C for 1 h in 8.6% glyoxal, 72% DMSO, and 14 mM phosphate buffer, pH 6.5, electrophoresed in a 1.2% agarose gel, blotted onto nylon membranes, and then hybridized with the $^{32}$P-labeled cDNA probes for 16 h at 60°C. Hybridized membranes were autoradiographed using Fuji RXU Medical X-Ray films (Fujifilm, Tokyo, Japan) at –80°C.

*Statistical analysis.* Paired t test was used to determine the differences in $PGE_2$ production between groups. The criterion for significance was $p < 0.05$.

## RESULTS

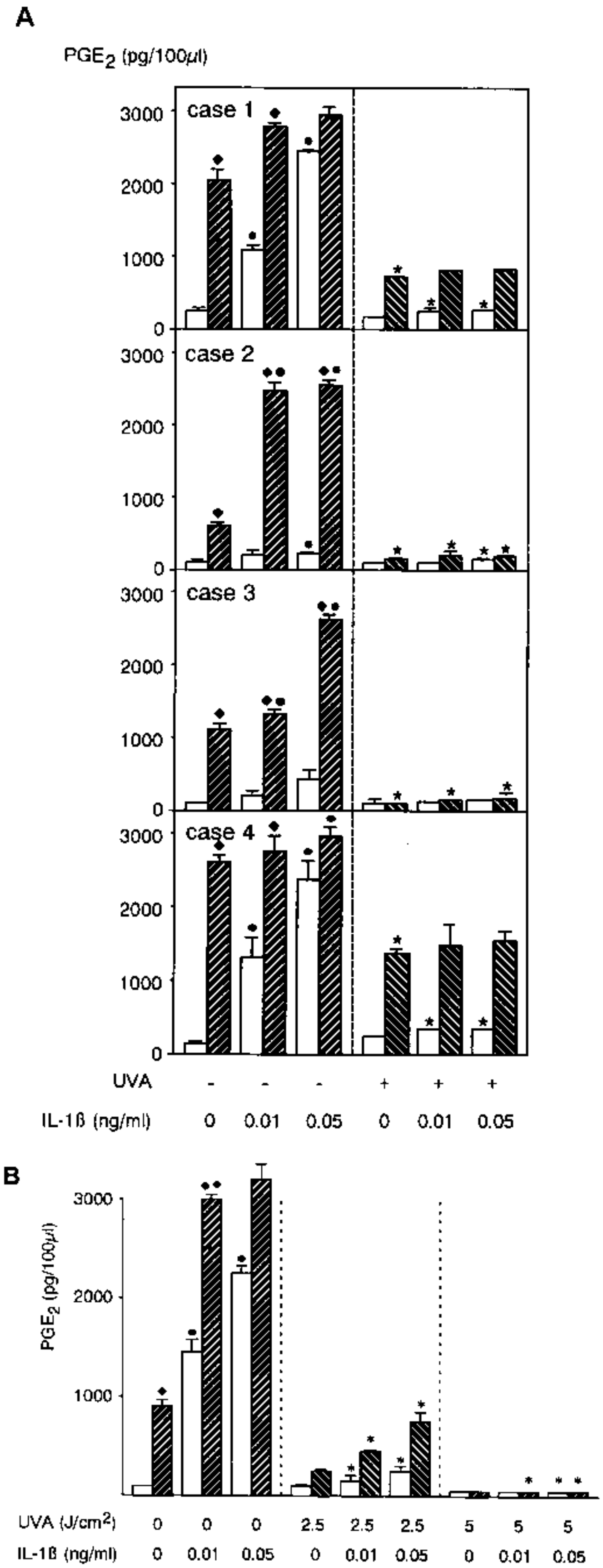
***$PGE_2$ production in fibroblasts from scleroderma and uninvolved skin*.** Constitutive and IL-1ß induced $PGE_2$ production was measured in fibroblasts from scleroderma and uninvolved skin of the 5 patients. As shown in Figure 1, IL-1ß induced a dose dependent increase in $PGE_2$ biosynthesis in fibroblasts from both scleroderma and uninvolved skin, but constitutive $PGE_2$ production was significantly greater in scleroderma fibroblasts than in fibroblasts from uninvolved skin, and IL-1ß induced $PGE_2$ production was also higher in scleroderma.

To verify that fibroblasts from uninvolved skin can be regarded as normal control samples, $PGE_2$ production in fibroblasts from involved skin, uninvolved skin, and the skin of healthy subjects was compared. There was a statistically significant difference in $PGE_2$ production between fibroblasts from scleroderma involved skin and the skin from healthy subjects, as well as between fibroblasts from scleroderma involved and uninvolved skin; the difference in $PGE_2$ production between fibroblasts from the skin of healthy subjects and from uninvolved skin was not significant (Table 1). Fibroblasts obtained from healthy subjects responded to IL-1ß treatment and UVA irradiation in the same manner as fibroblasts from uninvolved skin (data not shown). These results indicate that these responses are not specific to scleroderma patients and the different responses of scleroderma fibroblasts and fibroblasts from uninvolved skin are important in the development of this disorder.

*Expression of $cPLA_2$, COX-1, and COX-2 proteins in normal and scleroderma fibroblasts*. Using Western immunoblots, we examined the expression of $cPLA_2$, COX-1, and COX-2 in normal and scleroderma fibroblasts in the absence or presence of IL-1ß. When fibroblasts were treated with 0.05 ng/ml IL-1ß for 8 h, $cPLA_2$, COX-1, and COX-2 protein expression was induced in both normal and scleroderma fibroblasts. However, the expression level of $cPLA_2$ and COX-1 proteins was not altered in scleroderma fibroblasts compared with the normal fibroblasts. Unlike $cPLA_2$ and COX-1, both constitutive and IL-1ß induced COX-2 protein expression were enhanced in scleroderma fibroblasts (Figure 2A). The differences in the expression level of COX-2 correlated well with the amounts of $PGE_2$ produced by corresponding cells, as shown in Figure 1. These results indicate that COX-2 may be responsible for the enhanced biosynthesis of $PGE_2$.

*Expression of COX-2 mRNA in normal and scleroderma fibroblasts*. The expression of COX-2 mRNA was examined by Northern blotting. COX-2 mRNA expression was increased in response to the treatment with 0.05 ng/ml IL-1ß for 3 h in both normal and scleroderma fibroblasts, while both constitutive and IL-1ß induced COX-2 mRNA expression were upregulated in scleroderma fibroblasts (Figure 2B).

*Effects of UVA irradiation on $PGE_2$ biosynthesis and COX-2 expression*. We examined the effects of UVA irradiation on IL-1ß induced $PGE_2$ biosynthesis and COX-2 expression. When fibroblasts were pretreated with 5 J/cm$^2$ UVA irradia-



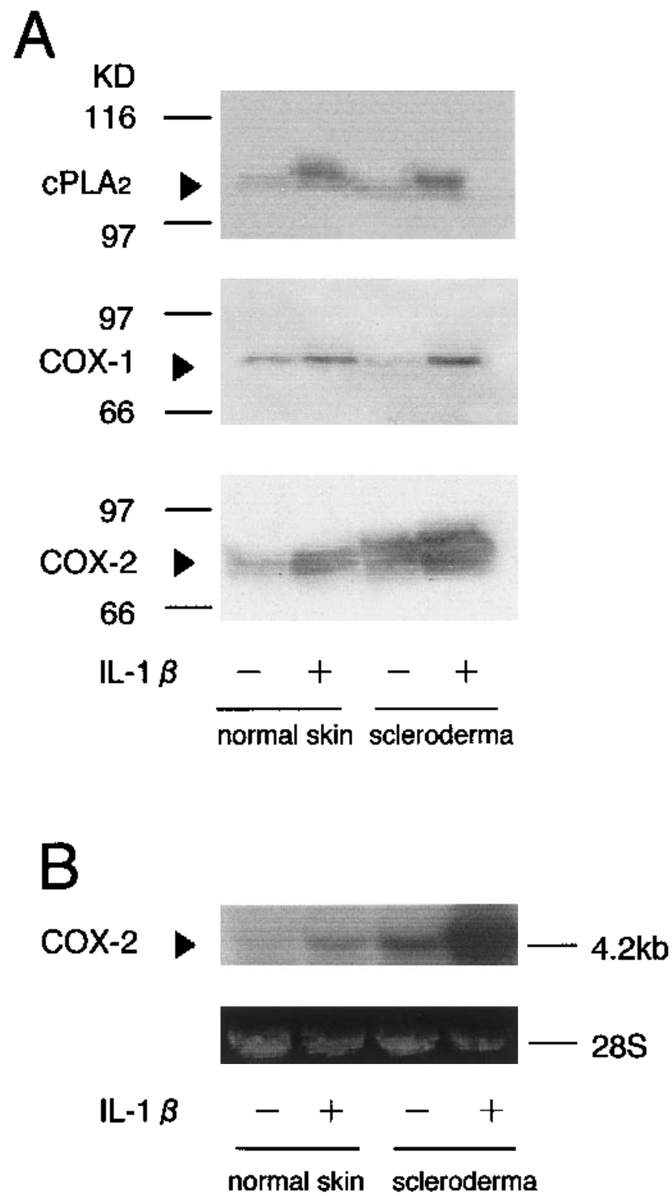




*Figure 2.* A. Proteins were extracted from confluent fibroblasts from normal skin and from scleroderma involved skin with no treatment or 0.05 ng/ml IL-1ß treatment for 8 h, and analyzed by Western immunoblotting using anti-COX-1, anti-COX-2, or anti-cPLA$_2$ antibodies. Twenty micrograms of protein were loaded per lane. B. Total RNA was extracted from confluent fibroblasts from normal skin and scleroderma involved skin with no treatment or 0.05 ng/ml IL-1ß treatment for 3 h, and analyzed by Northern blotting using $^{32}$P-labeled COX-2 probe. Twenty micrograms of RNA were loaded per lane.

←

*Figure 1.* Confluent fibroblasts from scleroderma involved (shaded bars) and normal skin (white bars) were treated with IL-1ß at the indicated concentrations for 24 h and amount of PGE$_2$ production was analyzed as described in Materials and Methods before or after UVA irradiation. Data are means ± SE from triplicate determinations of 2 separate experiments for each case. ◆: $p < 0.05$ vs fibroblasts from normal skin identically treated with IL-1ß. ●: $p < 0.05$ vs identical cells without IL-1ß treatment. *$p < 0.05$ vs identically treated counterparts without UVA irradiation.

*Table 1.* Comparison of $PGE_2$ production by fibroblasts from scleroderma involved skin, uninvolved skin, and skin from healthy subjects.

| | $PGE_2$ (pg/100 $\mu$l) | | |
|---|---|---|---|
| Fibroblasts from: | | | |
| Involved skin, n = 5 | 1466.6 ± 391.6 | p = 0.0218 | |
| Uninvolved skin, n = 5 | 136.2 ± 28.9 | | p = 0.0438 |
| Healthy subjects, n = 4 | 97.5 ± 2.5 | p = 0.3410 | |

Data shown are means ± SE of triplicate determinations of each case.

tion, $PGE_2$ biosynthesis induced by IL-1ß was inhibited (Figure 1). A dose dependent inhibition of $PGE_2$ biosynthesis by UVA treatment was observed in case 5 (Figure 1B). IL-1ß induced expression of both COX-2 mRNA and protein were also clearly inhibited by 5 J/cm$^2$ UVA irradiation (Figures 3A, 3B). Cells were not injured by 5 J/cm$^2$ UVA irradiation.

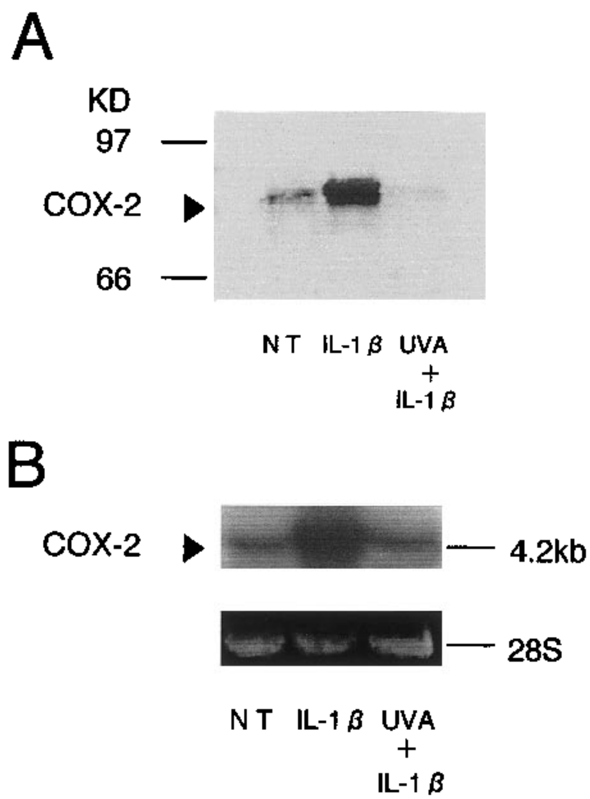



*Figure 3.* A. Proteins were extracted from confluent fibroblasts from scleroderma involved skin with no treatment (NT), 0.05 ng/ml IL-1ß treatment for 8 h (IL-1ß), and 0.05 ng/ml IL-1ß treatment for 8 h after 5 J/cm$^2$ UVA irradiation (UVA + IL-1ß) and analyzed by Western immunoblotting using anti-COX-2 antibody. Twenty micrograms of protein were loaded per lane. B. Total RNA was extracted from confluent fibroblasts from scleroderma involved skin with no treatment (NT), 0.05 ng/ml IL-1ß treatment for 3 h (IL-1ß), and 0.05 ng/ml IL-1ß treatment for 3 h after 5 J/cm$^2$ UVA irradiation (UVA + IL-1ß) and analyzed by Northern blotting using $^{32}$P-labeled COX-2 probe. Twenty micrograms of RNA were loaded per lane.

## DISCUSSION

Our study revealed that constitutive and IL-1ß induced $PGE_2$ production were greater in scleroderma than in normal fibroblasts. This is consistent with the observations that IL-1 signal transduction through the IL-1 receptor was induced excessively in scleroderma fibroblasts[3,4], suggesting that altered IL-1 mediated signal transduction in fibroblasts is involved in the pathogenesis of scleroderma. Because $PGE_2$ biosynthesis is regulated by 3 rate limiting enzymes ($cPLA_2$, which releases arachidonic acid from cell membranes to the cytoplasm, and COX-1 and 2, which convert arachidonic acid to prostaglandins), we examined the effects of IL-1ß on the expression of these enzymes in normal and scleroderma fibroblasts. While $cPLA_2$ and COX-1 protein expression were increased in response to IL-1ß treatment in both normal and scleroderma fibroblasts, the expression level did not increase in scleroderma fibroblasts compared with normal fibroblasts. However, unlike $cPLA_2$ and COX-1, both constitutive and IL-1ß induced COX-2 expression were significantly enhanced in scleroderma fibroblasts. COX-2 is known as the inducible enzyme, whose expression is induced by a wide variety of extracellular stimuli as part of the inflammatory response, including in connective tissue diseases[13,14]. It has been shown that COX-2 expression is increased by IL-1 stimulation in the synovial tissue from patients with rheumatoid arthritis[15,16], but this is the first report of enhanced expression of COX-2 in scleroderma. Using Western and Northern blotting, we demonstrated that COX-2 expression was regulated by both transcriptional and translational mechanisms.

We and others reported that PUVA therapy was beneficial for treating scleroderma[8-10]. While UVA and PUVA are not strictly equivalent, the effect of PUVA therapy is essentially based on UVA. Thus we examined the effects of UVA irradiation on IL-1ß induced COX-2 expression and $PGE_2$ biosynthesis. When fibroblasts were irradiated with UVA, both COX-2 expression and $PGE_2$ biosynthesis induced by IL-1ß were inhibited significantly. While UVA inhibited COX-2 expression transcriptionally and translationally, protein expression was decreased more than mRNA expression. This difference might depend on the time of IL-1ß treatment. Since we observed[17] that IL-1ß induced expression of COX-2 mRNA and protein attained the maximum in 3 and 8 h, respectively, RNA and protein were prepared after the treatment with IL-1ß for 3 and 8 h in this study.

Previous studies showed that an increase in $PGE_2$ is associated with suppression of collagen production in fibroblasts[18], whereas studies by Mauviel, *et al* indicated that collagen synthesis was independent from $PGE_2$ metabolism. In their studies, inhibition of collagen synthesis occurred concomitantly with increased secretion of $PGE_2$; however, blocking $PGE_2$ biosynthesis with indomethacin, a potent inhibitor of COX activity, did not counteract the inhibition of collagen synthesis in dermal fibroblasts treated with cytokines[19,20]. COX-2 is a bifunctional enzyme possessing COX activity that converts arachidonic acid to $PGG_2$ and peroxidase activity that generates $PGH_2$, a direct precursor of $PGE_2$, from $PGG_2$. In addition to its role in prostaglandin synthesis, the peroxidase activity of COX-2 contributes to alterations in intracellular redox status[21] that are associated with collagen metabolism[22]. Indomethacin inhibits the COX activity but not the peroxidase activity of COX-2[21]. Together with our findings, these facts suggest that inhibition of COX-2 expression is important in the suppression of collagen synthesis and that one mechanism by which PUVA therapy is beneficial in treating scleroderma may be by suppressing COX-2 expression, which results in the alteration of redox status and subsequent inhibition of collagen synthesis. Indeed, a previous study revealed that collagen synthesis in dermal fibroblasts was decreased in response to UV exposure[23].

We demonstrated that constitutive and IL-1ß induced $PGE_2$ biosynthesis is induced in scleroderma fibroblasts, that COX-2 is responsible for regulating $PGE_2$ biosynthesis, and that both COX-2 expression and $PGE_2$ biosynthesis were inhibited by UVA irradiation. Based on these observations, we propose that the novel therapeutic effects of PUVA therapy in treating scleroderma may result, at least in part, from the inhibition of COX-2 expression.

## REFERENCES

1. LeRoy EC. Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast. J Clin Invest 1974;54:880-9.
2. Jimenez SA, Feldman G, Bashey RI, Bienkowski R, Rosenbloom J. Co-ordinate increase in the expression of type I and type III collagen genes in progressive systemic sclerosis fibroblasts. Biochem J 1986;237:837-43.
3. Kawaguchi Y, Harigai M, Hara M, et al. Increased interleukin 1 receptor, type I, at messenger RNA and protein level in skin fibroblasts from patients with systemic sclerosis. Biochem Biophys Res Com 1992;184:1504-10.
4. Kawaguchi Y, Harigai M, Suzuki K, et al. Interleukin 1 receptor on fibroblasts from systemic sclerosis patients induces excessive functional responses to interleukin 1ß. Biochem Biophys Res Com 1993;190:154-61.
5. Raz A, Wyche A, Siegel N, Needleman P. Regulation of fibroblast cyclooxygenase synthesis by interleukin-1. J Biol Chem 1988;263:3022-8.
6. Ballou LR, Chao CP, Holness MA, Barker SC, Raghow R. Interleukin-1-mediated PGE2 production and sphingomyelin metabolism. Evidence for the regulation of cyclooxygenase gene expression by sphingosine and ceramide. J Biol Chem 1992;267:20044-50.
7. Korn JH, Torres D, Downie E. Clonal heterogeneity in the fibroblast response to mononuclear cell derived mediators. Arthritis Rheum 1984;27:174-9.
8. Kerscher M, Volkenandt M, Meurer M, Lehmann P, Plewig G, Rocken M. Treatment of localised scleroderma with PUVA bath photochemotherapy. Lancet 1994;343:1233.
9. Kanekura T, Fukumaru S, Matsushita S, Terasaki K, Mizoguchi S, Kanzaki T. Successful treatment of scleroderma with PUVA therapy. J Dermatol 1996;23:455-9.
10. Yamaguchi K, Takeuchi I, Yoshii N, Gushi A, Kanekura T, Kanzaki T. The discrepancy in hardness between clinical and histopathological findings in localized scleroderma treated with PUVA. J Dermatol 1998;25:544-6.
11. Ballou LR, Barker SC, Postlethwaite AE, Kang AH. Sphingosine potentiates IL-1-mediated prostaglandin E2 production in human fibroblasts. J Immunol 1990;145:4245-51.
12. Kanekura T, Laulederkind SJF, Kirtikara K, Goorha S, Ballou LR. Cholecalciferol induces prostaglandin E2 biosynthesis and transglutaminase activity in human keratinocytes. J Invest Dermatol 1998;111:634-9.
13. Herschman HR. Prostaglandin synthase 2. Biochim Biophys Acta 1996;1299:125-40.
14. Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 1996;271:33157-60.
15. Crofford LJ, Wilder RL, Ristimaki AP, et al. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1ß, phorbol ester, and corticosteroids. J Clin Invest 1994;93:1095-101.
16. Siegle I, Klein T, Backman JT, Saal JG, Nusing RM, Fritz P. Expression of cyclooxygenase 1 and cyclooxygenase 2 in human synovial tissue: Differential elevation of cyclooxygenase 2 in inflammatory joint diseases. Arthritis Rheum 1998;41:122-9.
17. Kirtikara K, Laulederkind SJ, Raghow R, Kanekura T, Ballou LR. An accessory role for ceramide in interleukin-1ß induced prostaglandin synthesis. Mol Cell Biochem 1998;181:41-8.
18. Clark JG, Kostal KM, Marino BA. Modulation of collagen production following bleomycin-induced pulmonary fibrosis in hamsters. Presence of a factor in lung that increases fibroblast prostaglandin E2 and cAMP and suppresses fibroblast proliferation and collagen production. J Biol Chem 1982;257:8098-105.
19. Mauviel A, Redini F, Hartmann DJ, Pujol JP, Evans CH. Modulation of human dermal fibroblast extracellular matrix metabolism by the lymphokine leukoregulin. J Cell Biol 1991;113:1455-62.
20. Mauviel A, Heino J, Kahari VM, et al. Comparative effects of interleukin-1 and tumor necrosis factor-a on collagen production and corresponding procollagen mRNA levels in human dermal fibroblasts. J Invest Dermatol 1991;96:243-9.
21. Subbaramaiah K, Telang N, Ramonetti JT, et al. Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res 1996;56:4424-9.
22. Hernandez Munoz R, Diaz Munoz M, Chagoya de Sanchez V. Possible role of cell redox state on collagen metabolism in carbon tetrachloride-induced cirrhosis as evidenced by adenosine administration to rats. Biochim Biophys Acta 1994;1200:93-9.
23. Shindo Y, Hashimoto T. Antioxidant defence mechanism of the skin against UV irradiation: study of the role of catalase using acatalasaemia fibroblasts. Arch Dermatol Res 1995;287:747-53.