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. 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. It was reported that fibroblasts produced prostaglandin E2 (PGE2) in response to IL-1 treatment, and possible correlation between PGE2 and collagen synthesis in scleroderma was discussed. 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). 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 involved skin samples. 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 18 (IL-18) mediated signal transduction in scleroderma fibroblasts and those from normal skin.

Methods. Prostaglandin E2 (PGE2) production and expression of cytosolic phospholipase A2 (cPLA2), cyclooxygenase (COX)-1, and COX-2 (enzymes that regulate PGE2 production) were examined in untreated and IL-18 treated fibroblasts from scleroderma involved and normal skin. The effect of UVA irradiation on enzyme expression and PGE2 production was examined. PGE2 was measured by a competitive radioimmunoassay and enzyme expression was analyzed by Western immunoblotting and Northern blotting.

Results. Constitutive PGE2 production was significantly upregulated and IL-18 induced PGE2 production was increased by the enhancing expression of both COX-2 mRNA and protein in fibroblasts from scleroderma involved skin; PGE2 production and COX-2 expression were inhibited by UVA irradiation.

Conclusion. Enhanced PGE2 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 PGE2 production.
Cell culture. Dermal fibroblasts were cultured as described\textsuperscript{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/ml penicillin G, 100 mg/ml streptomycin, 1 mg/ml Fungizone, 0.1 mM nonessential amino acids, 292 mg/l glutamine, 50 mg/ml ascorbic acid, and 10% fetal calf serum under sterile conditions at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2}. The media were changed every 3 days until cells became confluent (4–6 wks), then cells were seeded at 10\textsuperscript{5} cells/ml in identical medium in 24 well flat bottom tissue culture plates for PGE\textsubscript{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\textsuperscript{2}.

PGE\textsubscript{2} measurement. PGE\textsubscript{2} was measured by radioimmunooassay\textsuperscript{12}. The method is based on the competition of PGE\textsubscript{2} (DuPont-NEN, Boston, MA, USA) for binding to anti-PGE\textsubscript{2} antibody (Upstate Biotechnology, Lake Placid, NY, USA). Cells were seeded at 1 × 10\textsuperscript{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-1B at appropriate concentrations. A 10 to 100 µl 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\textsubscript{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\textsubscript{2} in the samples were calculated.

Western immunoblotting. Fibroblasts from scleroderma and uninvolved skin were seeded at 1 × 10\textsuperscript{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 µg/ml leupeptin, 0.03% Aprotinin, 2 mM PMSF, and 2 mM EDTA, pH 7.2. Proteins were extracted by suspending the pellet in PBS containing 0.1% sodium azide, and 0.1% gelatin), mixed with the appropriate amounts of labeled PGE\textsubscript{2} 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\textsubscript{2} in the samples were calculated.

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 6% 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 \textsuperscript{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\textsubscript{2} production between groups. The criterion for significance was p < 0.05.

RESULTS

PGE\textsubscript{2} production in fibroblasts from scleroderma and uninvolved skin. Constitutive and IL-1B induced PGE\textsubscript{2} produc-
Figure 1. Confluent fibroblasts from scleroderma involved (shaded bars) and normal skin (white bars) were treated with IL-1β at the indicated concentrations for 24 h and amount of PGE₂ production was analyzed as described in Materials and Methods before or after UV A 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-1β. ● p < 0.05 vs identical cells without IL-1β treatment. *p < 0.05 vs identically treated counterparts without UV A irradiation.

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-1β treatment for 8 h, and analyzed by Western immunoblotting using anti-COX-1, anti-COX-2, or anti-cPLA₂ 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-1β treatment for 3 h, and analyzed by Northern blotting using ³²P-labeled COX-2 probe. Twenty micrograms of RNA were loaded per lane.
tion, PGE₂ biosynthesis induced by IL-1ß was inhibited (Figure 1). A dose dependent inhibition of PGE₂ 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/cm² UVA irradiation (Figures 3A, 3B). Cells were not injured by 5 J/cm² UVA irradiation.

**DISCUSSION**

Our study revealed that constitutive and IL-1ß induced PGE₂ 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 fibroblasts³,⁴, suggesting that altered IL-1 mediated signal transduction in fibroblasts is involved in the pathogenesis of scleroderma. Because PGE₂ biosynthesis is regulated by 3 rate limiting enzymes (cPLA₂, 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 cPLA₂ 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 cPLA₂ 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 diseases¹³,¹⁴. It has been shown that COX-2 expression is increased by IL-1 stimulation in the synovial tissue from patients with rheumatoid arthritis¹⁵,¹⁶, 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 scleroderma⁸-¹⁰. 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 PGE₂ biosynthesis. When fibroblasts were irradiated with UVA, both COX-2 expression and PGE₂ 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 observed¹⁷ that IL-1ß induced expres-

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<th>Fibroblasts from:</th>
<th>PGE₂ (pg/100 µl)</th>
<th>p</th>
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<tr>
<td>Involved skin, n = 5</td>
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<tr>
<td>Uninvolved skin, n = 5</td>
<td>136.2 ± 28.9</td>
<td>0.3410</td>
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<tr>
<td>Healthy subjects, n = 4</td>
<td>97.5 ± 2.5</td>
<td>0.0438</td>
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Data shown are means ± SE of triplicate determinations of each case.

**Table 1.** Comparison of PGE₂ production by fibroblasts from scleroderma involved skin, uninvolved skin, and skin from healthy subjects.

**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/cm² 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/cm² UVA irradiation (UVA + IL-1ß) and analyzed by Northern blotting using ³²P-labeled COX-2 probe. Twenty micrograms of RNA were loaded per lane.

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sion 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-1β 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	extsuperscript{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	extsuperscript{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 synthesis in dermal fibroblasts was decreased in response to alteration of redox status and subsequent inhibition of collagen synthesis, the peroxidase activity of COX-2 contributes to synthesis, the peroxidase activity of COX-2 contributes to

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	extsuperscript{23}.

We demonstrated that constitutive and IL-1β 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