Uroporphyrin I Stimulation of Collagen Biosynthesis in Human Skin Fibroblasts

A UNIQUE DARK EFFECT OF PORPHYRIN

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ABSTRACT Porphyria cutanea tarda and erythropoietic porphyria are disorders of heme synthesis that originate in the liver and bone marrow, respectively. Each is characterized by increased accumulation of uroporphyrin, I, by cutaneous photosensitivity, and in some patients by indurated plaques and scarring that resemble scleroderma. These scleroderma-like lesions occur in light-exposed and light-protected body areas. In these studies we evaluated the role of uroporphyrin I and of light in evoking the scleroderma-like cutaneous changes. Normal human skin fibroblasts were exposed to uroporphyrin I and to 400 nm radiation and the effect of these agents on collagen accumulation by the cells was determined. Radioactive tracer studies showed that uroporphyrin I caused a specific increase in the accumulation of newly synthesized collagen by fibroblast monolayer cultures, as verified by [^{3}H]hydroxyproline and collagenase digestion assays. Collagen accumulation was stimulated 1.5- to 2.7-fold by uroporphyrin I, whereas noncollagenous protein accumulation was unchanged. The increased collagen accumulation was time and uroporphyrin I-concentration-dependent, and occurred both in the presence or absence of ultraviolet light exposure. Further studies demonstrated that the increased accumulation was not the result of decreased rates of collagen degradation nor was it due to changes in cell population growth parameters (generation times and saturation densities). No changes in morphology of the treated cells occurred. These studies indicate that porphyrins possess previously undemonstrated biological effects that are independent of their photosensitizing properties. This novel dark effect of uroporphyrin I may account for the sclerodermatous lesions seen in the skin of patients with porphyria cutanea tarda and erythropoietic porphyria.

INTRODUCTION

The porphyrias are inherited or acquired disorders of heme synthesis in the bone marrow or the liver (1). These diseases are characterized by clinical syndromes thought to be secondary to the toxic effects of porphyrins or porphyrin precursors that are synthesized and accumulate in excess. In each of the porphyrias there is an identifiable pattern of abnormal porphyrin or porphyrin precursor excretion in the urine or feces that usually permits diagnosis in the majority of patients. Those porphyrias in which excessive porphyrins accumulate are usually characterized by cutaneous photosensitivity that develops as a consequence of the absorption of radiant energy by porphyrins ~400 nm (the Soret band). When this photosensitizing reaction occurs in the skin, photosensitivity results.

In porphyria cutanea tarda, excessive uroporphyrin I is found in the urine, plasma, feces, and skin of patients (2–5). The increased porphyrin accumulation is apparently due to deficient activity of the hepatic enzyme uroporphyrinogen decarboxylase that may be inherited or acquired (6–8). An increase in hepatic iron stores and ingestion of and exposure to certain drugs and chemicals are also important factors in the pathogenesis of this disease (9–11). The changes in the light-exposed skin of patients with porphyria cutanea tarda

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(PCT)[1] include increased bruisability, subepidermal vesicles and bullae, facial hypertrichosis, and pigmentary changes (12). Furthermore, in ~25% of patients with PCT, indurated hypopigmented plaques often indistinquishable from the cutaneous lesions of scleroderma are seen. Unlike the blistering lesions of PCT, which are due to cutaneous photosensitivity, these sclerodermatous plaques are found in both light-exposed and light-protected body areas (13, 14). This suggests that photosensitivity is not an absolute requirement for this pathologic reaction to occur. Similar skin changes as well as a severe mutilating photosensitivity occur in patients with erythropoietic porphyria (EP)(Günthers disease). This type of porphyria is characterized by excessive accumulation of uroporphyrin I, which originates in the bone marrow.

Ultrastructural studies have shown that these indurated cutaneous plaques are similar to those observed in scleroderma (15). The thickness of the dermis is increased due to collagen deposition and the individual collagen fibrils are smaller and display large variations in cross-sectional diameter (15). Because the turnover of collagen in human skin in vivo is quite slow and yet sclerodermatous plaques seem to appear rapidly, it is likely that the dermal fibroblasts within these lesions have been stimulated to form increased amounts of collagen. The present study was therefore undertaken to assess the effects of uroporphyrin I and of light on collagen synthesis in cultured normal human skin fibroblasts.

METHODS

Cell culture techniques. Fibroblasts were obtained from explant cultures of normal adult human skin. Monolayer cultures were maintained in 30 or 60-mm Falcon plastic tissue culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) and used for experiments only during the 5th to 10th passage. The culture medium was Ham's F-10 supplemented with 10% fetal calf serum, twice the normal concentrations of amino acids and sodium pyruvate, 2.5 μg/ml amphotericin B, 100 μg/ml streptomycin, 100 U/ml penicillin, and 60 μg/ml Tylocine. Cultures were maintained at 37°C in a humid atmosphere containing 5% CO_2/95% air. To determine cell growth parameters, cells in monolayer culture were trypsinized (0.01% trypsin, Worthington Biochemical Corp., Freehold, N. J., + 1% EDTA in phosphate-buffered saline) at various times during the growth cycle and counted using a hemocytometer. Generation times (G_t) were calculated from the growth curve slopes during the logarithmic phase, according to the equation: G_t = h in culture/number of divisions, where number of divisions = (log final cell number − log initial cell number)/log 2. Saturation densities were determined after 10 d and expressed as cells per square centimeter of culture surface.

Uroporphyrin I preparation and ultraviolet light irradiation. Crystals of uroporphyrin I (Porphyrin Products, Logan, Utah) were dissolved in 2 N HCl, neutralized with NaOH, and the solution desalted by passing over a 1.5 × 20-cm Sephadex G-10 column. The column was eluted with distilled water and the solubilized porphyrin collected and sterilized by passing through a 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.). The concentration of uroporphyrin I in this stock solution was calculated using an extinction coefficient E_{mM} = 541 at 415.5 nm in 0.5 N HCl (16). The stock solution was mixed with fetal calf serum, and added to the culture medium to obtain the desired concentration of uroporphyrin I. Fibroblast monolayers were irradiated at room temperature using a high-pressure mercury arc lamp with a Wood's filter (Ultraviolet Products, Inc., San Gabriel, Calif.). The irradiance of this light source was 2 mW/cm^2 (wavelengths 380–430 nm) and the dose of light ranged from 1.6 to 3.2 J/cm^2. In all experiments involving uroporphyrin I the culture medium containing the porphyrin was added to the cells in the dark and allowed to equilibrate for 24 h. This preincubation was used because of the prior studies of Schothorst et al. (17), which showed that porphyrin uptake into cultured fibroblasts occurs slowly (12–96 h), and that photosensitivity is enhanced by preincubation. The culture medium was then removed and the cell layers rinsed twice with Hanks' balanced salt solution without phenol red. In the experiments involving light as a variable, the appropriate cultures were then irradiated. All manipulations were performed in a photographic dark room using only a red safety light for illumination and the light-protected cultures were wrapped with double layers of heavy aluminum foil.

Collagen and noncollagen protein synthesis assays. Cell monolayers were preincubated for 24 h in the dark with culture medium containing uroporphyrin I to insure adequate uptake of the chemical. Triplicate cultures were then incubated for appropriate times in F-10 medium (with or without uroporphyrin I), which lacked [^{12}C]proline but contained 2 μCi/ml [^{3}H]proline (New England Nuclear NET-285, New England Nuclear, Boston, Mass.), and 50 μg/ml ascorbic acid. After incubation, the combined cells and medium were precipitated with 5% TCA + 0.25% tannic acid and dialyzed for 48 h against running tap water to remove free radioactivity. The TCA-tannic acid precipitate was hydrolyzed in 6 N HCl for 24 h at 110°C, and the radioactivity in hydroxyproline and proline was then determined using a chemical assay (18, 19). Some data are expressed as % hypro = disintegrations per minute hydroxyproline/(disintegrations per minute hydroxyproline + disintegrations per minute proline) × 100. Since it is assumed that only collagen or collagen derivatives contain significant hydroxyproline, this value is an estimate of the amount of collagen synthesized relative to all proline-containing proteins. Radioactivity in collagenous and noncollagenous proteins was calculated as described previously (20). Since collagen contains ~110 residues of proline and 100 residues of hydroxyproline per alpha chain, the total proline-derived radioactivity in collagen is 2.1 times the hydroxyproline. Subtraction of this value from the total radioactivity gives an estimate of the noncollagenous protein. Content of newly synthesized collagen using a collagenase assay was performed as described by Peterkofsky and Diegelmann (21) using clostridial collagenase (CLSPA, Worthington Biochemical Corp.).

Collagen degradation. A "pulse-chase" experiment was performed to determine the turnover rates of the newly-synthesized collagen. Fibroblast cultures were "pulsed" for 40 h with [^{3}H]proline, at which time the cell layers were rinsed at 37°C with Hanks' balanced salt solution (four changes at 15-min intervals) and fresh F-10 medium with

[1] *Abbreviations used in this paper:* EPP, erythropoietic protoporphyria; EP, erythropoietic porphyria; PCT, porphyria cutanea tarda.

or without 25 μM uroporphyrin I was added. At 12-h intervals during the ensuring 3-d "chase period", the media from triplicate cultures were collected and precipitated with TCA-tannic acid (5–0.25% final concentration). After centrifugation, the supernatants and pellets were hydrolyzed with 6 N HCl and analyzed for content of [^{3}H]hydroxyproline using 0.9 × 12-cm columns of Dowex 50 resin (Dow Corning Corp., Midland, Mich.) (22).

RESULTS

Effects of uroporphyrin I on collagen and noncollagen protein accumulation. Fibroblast monolayers were preincubated for 24 h in the dark with or without uroporphyrin I (25 μM) and then incubated for 24 h with [^{3}H]proline. Accumulation of nondialyzable isotopically labeled hydroxyproline increased 2.7-fold when uroporphyrin I was included in the incubation medium (Table I). This represented a specific increase in collagen synthesis, as noncollagenous protein accumulation in the same cultures decreased slightly in this experiment. Increased collagen synthesis was also demonstrated using the collagenase assay. A twofold increase in % hypro values was paralleled by a 1.8-fold increase in collagenase digestibility of newly synthesized protein (Table II). The cells and media were separately analyzed for isotopically labeled proline and hydroxyproline. Because no differences in partitioning were seen in control and uroporphyrin I-treated cultures, the data presented in Tables I and II were each combined and are presented on a per culture basis in each table.

Kinetics of uroporphyrin I-stimulated collagen accumulation. The uroporphyrin I-induced increase in collagen accumulation was time dependent and occurred either in the presence or absence of ultraviolet light (Fig. 1). After preincubation of cells with the porphyrin for 24 h, [^{3}H]proline was added to the cultures, which were then harvested at 6-h intervals during the ensuing 24 h. At each time point there was a 1.5- to 2-fold increase in collagen accumulation in the uroporphyrin I-treated cultures as compared to controls (light without porphyrin and dark without porphyrin). It is important to emphasize that the stimulating effect of uroporphyrin I occurred regardless of light exposure indicating that this was not the result of a photosensitizing reaction. The effect of uroporphyrin I on collagen accumulation was also concentration-dependent up to a concentration of 25 μM (Fig. 2).

Effects of uroporphyrin I on collagen turnover. A pulse-chase experiment was performed to assess the possibility that the increased collagen accumulation in the presence of uroporphyrin I might be the result of decreased collagen degradation. Replicate cultures were pulsed for 40 h with [^{3}H]proline (without uroporphyrin I) at which time the radioactive medium was removed and replaced with fresh, nonradioactive medium with or without the porphyrin. Medium was then collected at 12-h intervals during the ensuing 72-h chase period and assayed for isotopically labeled collagen. Radioactive collagen was released into the medium during the chase period and the rate of release was similar in the presence or absence of uroporphyrin I (Fig. 3, upper panel). The TCA-soluble fraction in the medium was similar for controls and uroporphyrin I-treated cultures (Fig. 3, lower panel). The TCA-soluble fraction provides an estimate of the extent of cellular degradation of collagen that is initiated by collagenase (23). These results indicate that the increase in collagen accumulation induced by uroporphyrin I was not due to decreased turnover of the protein.

Effects of uroporphyrin I on cell growth. The effects of uroporphyrin I and light on the growth of human fibroblasts were examined. As shown in Fig. 4, the growth curves were similar for all cultures including the controls (no light, no porphyrin), light without porphyrin, porphyrin without light and por-

TABLE I

Effects of Uroporphyrin I on Collagen and Noncollagen Protein Synthesis by Human Skin Fibroblasts

Culture	Total radioactivity dpm/culture*	Hydroxyproline dpm/culture‡	% Hypro§	Collagen dpm/culture‖	Noncollagen dpm/culture¶
Control	2,354,840±147,406	32,967±7,064	1.4±0.3	69,231±4,333	2,285,609±143,072
25 μM uroporphyrin I	2,038,721±151,608	89,092±6,625	4.4±0.9	187,093±14,008	1,851,628±137,600

* Combined cells + medium.

‡ Hydroxyproline determined as described in Methods.

§ % Hypro = (dpm Hydroxyproline/Total dpm) × 100.

‖ Collagen radioactivity = 2.1 × hydroxyproline radioactivity.

¶ Noncollagen radioactivity = Total dpm − dpm in collagen.

Replicate logarithmic phase 60 mm monolayer cultures of human skin fibroblasts were preincubated 24 h in F-10 medium with or without 25 μM uroporphyrin I. The medium was removed and fresh medium containing [^{3}H]proline, with or without uroporphyrin I, was added. After 24 h the cells and medium were assayed for radioactive peptidyl proline and hydroxyproline. Each value represents the mean±SD from three cultures.

TABLE II

Comparison of the [^{3}H]Hydroxyproline Content and Collagenase Sensitivity of Labeled Proteins Synthesized by Control and Uroporphyrin I-Treated Human Skin Fibroblasts

Culture	dpm/culture	% Hypro	% Total dpm solubilized by collagenase
Control	246,203±16,285	2.4±0.4	7.9±1.9
25 μM uroporphyrin I	241,300±3,667	4.8±0.7	14.5±0.7

Replicate logarithmic-phase cultures were incubated with [^{3}H]proline as described in the legend to Table I. At the end of the incubation one-half the cultures were analyzed for labeled proline and hydroxyproline and one-half were assayed for digestibility with collagenase (Methods). Each value represents the mean±SD from three cultures.

phyrin irradiated daily with 400 nm light. The average generation time for all the cultures was calculated to be 30±5 h and the saturation densities 5 × 10^4±9, 950 cells/cm^2. There were no obvious differences in cellular morphology in the cultures when examined by phase-contrast microscopy.

DISCUSSION

The studies described here define a new biological effect of uroporphyrin I. This compound caused a 1.5- to 2.7-fold increase in the accumulation of newly synthesized collagen by monolayer cultures of normal human skin fibroblasts, whereas noncollagen protein synthesis was unaffected. This specific increase in collagen synthesis, as measured by two different assays, was dependent upon uroporphyrin I concentration, was time dependent and occurred in the presence or absence of exposure to 400 nm ultraviolet light. The possibility that the increased accumulation was caused by an effect of uroporphyrin I to decrease collagen degradation rates was ruled out because pulse-chase experiments showed no effect of the porphyrin on collagen turnover rates. The possibility was also considered that uroporphyrin I somehow interfered with the growth of fibroblasts, perhaps by restricting them during some phase of the cell cycle. Bosman (24) has demonstrated that the collagen glycosyl transferase en-

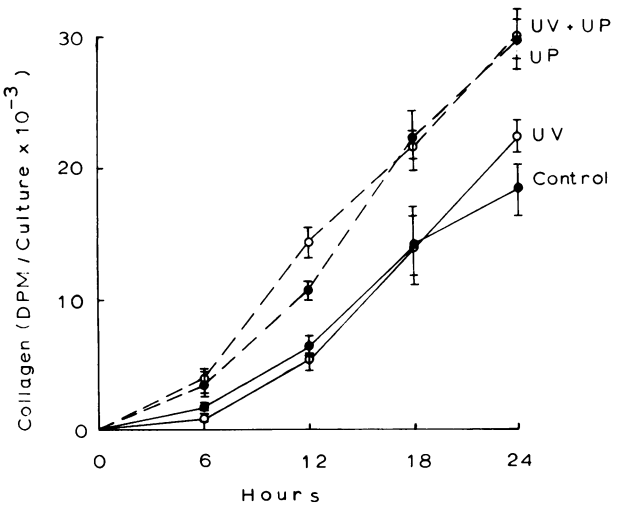

FIGURE 1 Time-dependent effects of uroporphyrin I and ultraviolet light on collagen accumulation by cultured human skin fibroblasts. Replicate cultures were preincubated 24 h with or without 25 μM uroporphyrin I. At that time labeling medium was added and the cultures harvested at 6-h intervals during the ensuing 24-h period and counts per minute were determined. The uroporphyrin-treated cultures (UP and UV + UP) were labeled in the presence of the porphyrin and the cultures treated with ultraviolet light (UV and UV + UP) were irradiated for 15-min intervals (1–3 J/cm^2) at the beginning of the pulses. The values represent the mean of three cultures.

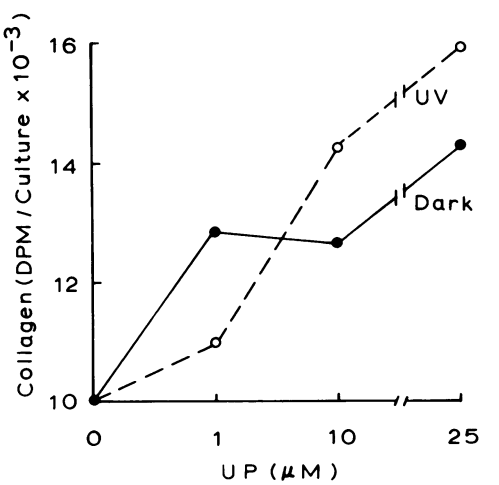

FIGURE 2 Concentration effects of uroporphyrin I on collagen synthesis by cultured human fibroblasts. Triplicate cultures were preincubated 24 h with the indicated concentration of uroporphyrin I. The media were then replaced with labeling medium containing uroporphyrin and [^{3}H]proline and assayed 24 h later for [^{3}H]hydroxyproline. The ultraviolet light-treated cultures (UV) were irradiated 15 min (1–3 J/cm^2) immediately before the start of the labeling period.

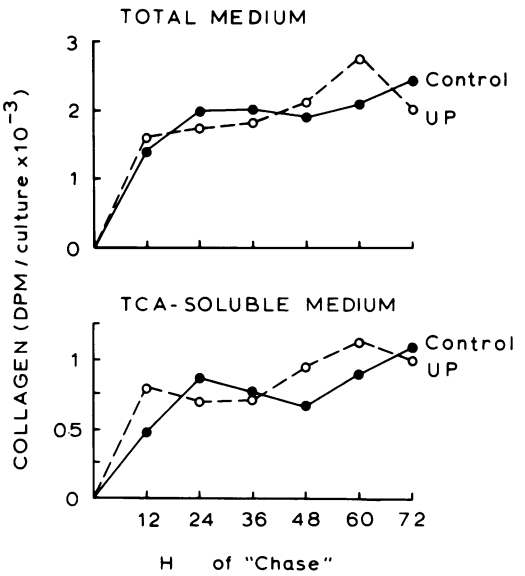

FIGURE 3 Effects of uroporphyrin I on collagen degradation rates. Replicate cultures were pulsed 40 h with [^{3}H]proline, rinsed and chased 72 h with medium lacking [^{3}H]proline with or without uroporphyrin I. At the indicated times during the chase period the medium from separate cultures was analyzed for TCA-insoluble collagen and TCA-soluble collagen (lower panel) as described in Methods. Total collagen (upper panel) was calculated by addition of the values from the two fractions.

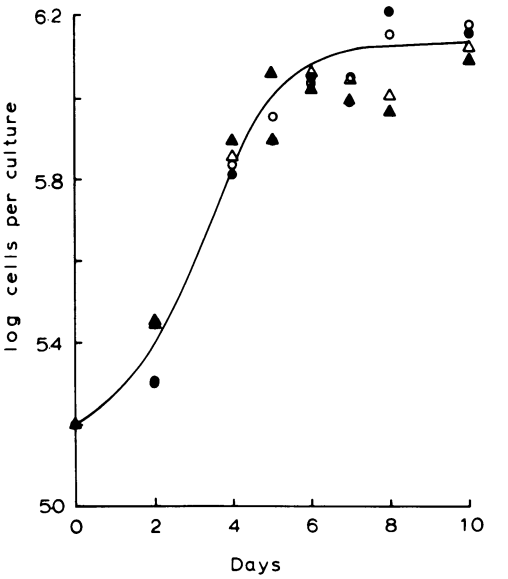

FIGURE 4 Effects of uroporphyrin I (UP) and ultraviolet light on fibroblast population growth. Triplicate 60-mm cultures of fibroblasts were trypsinized and counted at the indicated times. Uroporphyrin-treated cultures were grown continuously with 25 μM uroporphyrin I and ultraviolet light-treated cultures were irradiated 15 min (1–3 J/cm^2) each day. ●, control; ○, with UV; ▲, with UP; △, with UV and UP.

zymes, which posttranslationally modify collagen alpha chains, display peak activities during the S phase of the cell cycle, and Schiltz (25) recently showed that collagen synthesis in cultured chick cartilage cells was maximal during the S phase. Our experiments ruled out cell cycle effects since no gross differences in generation times or saturation densities occurred in cells to which uroporphyrin I was added. In summary, it is likely that the observed increase in collagen accumulation in uroporphyrin I-treated cells resulted from specifically increased rates of collagen biosynthesis.

Much information is available regarding the processes of synthesis, hydroxylation, glycosylation and secretion of procollagens, and the subsequent enzymatic cleavage of these larger precursor molecules into collagen gamma components which then form fibers (see references 26–28 for recent reviews). However, little is known about the specific regulation of collagen relative to noncollagen protein synthesis by connective tissue cells. Many reports have demonstrated modification of collagen synthesis by various factors and conditions (reviewed by Rojkind and Dunn [29]). For example, ascorbate has been shown to enhance collagen synthesis or to have no effect upon it (30, 31).

Clearly, the effects of uroporphyrin I observed here were specific for collagen synthesis. Since the discovery of procollagen in 1971 (32–35) it has been hypothesized that the amino or carboxy terminal peptides from procollagen, which are enzymatically generated in the extracellular spaces, might function to regulate collagen synthesis. Wiestner et al. (36) provided experimental support for this hypothesis by demonstrating that the amino-terminal peptides of types I and III procollagens specifically inhibited collagen synthesis by cultured fibroblasts (but not type II-producing cartilage cells). It is conceivable that uroporphyrin I may have stimulated collagen synthesis by interfering with this feedback mechanism, either by binding to the procollagen peptide(s) or to a cell surface peptide receptor.

The mechanism of tissue damage by porphyrins remains controversial. Early studies showed that treatment of monkey kidney cells in vitro with uroporphyrin I and 400 nm light resulted in lysosomal damage that in turn led to release of enzymes (proteases) with the potential to evoke membrane damage in tissue (37). Extensive studies in erythrocytes obtained from patients with erythropoietic protoporphyria (EPP) have shown that after irradiation with 400 nm light these protoporphyrin-laden cells undergo colloid osmotic hemolysis (38). Other studies have shown that lipid peroxidation occurs in these photohemolyzed erythrocytes (39).

Recent studies from our laboratories have shown

that treatment of cultured normal human skin fibroblasts with protoporphyrin and 400 nm light caused a time- and protoporphyrin-concentration dependent cytolysis during irradiation that was accompanied by decreased cellular viability and decreased rates of protein synthesis (40). Furthermore, the photosensitizing action was associated with insolubilization of cellular proteins suggesting that the phototoxic effect of protoporphyrin was related to light-induced denaturation of proteins.

The studies reported in this paper indicate that uroporphyrin I has a major stimulatory effect on collagen synthesis in cultured human fibroblasts and that this effect occurs in the presence or absence of 400 nm light. This contrasts with our prior data obtained with protoporphyrin IX in which phototoxic damage in cells was associated with a decrease in protein synthesis. These differential effects of uroporphyrin I and protoporphyrin IX in cultured human skin fibroblasts are of interest particularly since the photosensitizing effects of the different porphyrins vary considerably in the skin in vivo. In EPP, protoporphyrin IX is the predominant porphyrin and the disease is characterized by a cutaneous photosensitivity reaction that includes immediate painful erythema and edema. In PCT and in EP, uroporphyrin I is the predominant porphyrin and the disease is characterized by a photosensitivity reaction that includes blistering and increased skin fragility. Furthermore, PCT and EP patients develop indurated scarred areas of skin that occur in both light-exposed and light-protected regions. These skin changes closely resemble morphea or localized scleroderma. Ultrastructural studies have shown that the collagen content of these indurated plaques is increased and that the diameter of the collagen fibrils is similar to that observed in morphea (15). It is of interest that the scleroderma-like lesions of PCT resolve slowly once the disease is brought into remission and porphyrin levels diminish (13).

The results reported here indicating that uroporphyrin I, the major porphyrin present in excess in patients with PCT and EP, specifically stimulates collagen accumulation in normal human skin fibroblasts raises the possibility that porphyrins could possess additional biological effects that are independent of light. It is of interest that Lim and Gigli (41) have recently shown that at high doses (>5 μg/ml) protoporphyrin IX can decrease the activity of the C5 component of guinea pig complement in the absence of light.

The stimulatory effect of uroporphyrin I on collagen accumulation and the course of the disease in which the indurated plaques slowly improve if the excessive porphyrin accumulation returns to normal, suggest that the porphyrin itself is responsible for these biological effects. It should be of considerable interest to determine whether porphyrins have other biological effects independent of their photosensitizing properties.

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