Increased Oxidative Damage to Fibroblasts in Skin With and Without Lesions in Psoriasis

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Differences in oxidative damage, as measured by an increase in the carbonylation of macromolecules, were determined *in situ* with skin biopsies from psoriatic patients and controls. High levels of carbonyl residues were consistently detected in the dermis and never in the epidermis of sections of these skin biopsy samples. The dermis of psoriatic skin without lesions had a higher level of carbonylation than the dermis of normal skin. In this study, we found that there was more oxidative damage in cultured fibroblasts prepared from skin with and without lesions from psoriasis patients than in normal fibroblasts from the skin of age-matched controls. The extent of protein carbonylation in cell extracts was determined by immunoblotting, using an antidinitrophenylhydrazone antibody, and in intact cells was determined by immunocytochemical analysis with the same antibody. The higher level of carbonylation detected was used here as a measure of oxidative stress, and showed that some oxidative damage occurred before the appearance of typical psoriatic plaques. These results suggest that fibroblasts are affected before the onset of psoriasis and that this damage is independent of any inflammatory infiltrate. ***Key words: dinitrophenylhydrazine/fibroblasts/oxidative modification/protein carbonyls/psoriasis/skin. J Invest Dermatol 114:984–989, 2000***

Psoriasis is a common disease (Christophers and Sterry, 1993) (2% of the population) that generally presents as persistent plaques on the skin of the knees, elbows, or scalp; 5%-12% of psoriasis patients also develop chronic arthritis (Krueger and Duvic, 1994). The skin lesions are chronic, erythematous, hard, and scaly. Histopathologic studies have shown parakeratosis, microabscesses, the absence of a granular layer, elongation and edema of papillae, and an increase in the number of mitotic divisions of keratinocytes in the epidermis of psoriatic plaques. Dermal lesions typically involve an inflammatory infiltration of mononuclear cells into the upper dermis. This inflammatory infiltration precedes epidermal changes resulting in psoriatic plaques on the affected areas of skin (Christophers and Mrowietz, 1995). In contrast, areas of skin without lesions in psoriasis patients have epidermal hyperplasia only, and no inflammatory infiltration.

Psoriasis is an inherited, polygenic disease (Elder *et al*, 1994; Henseler, 1997). Recent studies have suggested a key role for T lymphocytes in the pathologic physiology of this disease, which implies that psoriasis may be an immunologic disorder (Bato-Csorgo *et al*, 1995; Gottlieb *et al*, 1995; Christophers, 1996). T cells ($CD4^+$ and $CD8^+$) and monocytes are detected early in the formation of psoriatic lesions, and these lymphocytes secrete cytokines, which stimulate keratinocyte proliferation (Elder, 1995) *in vitro* and *in vivo*. Immunosuppressants such as cyclosporine (Ellis *et al*, 1991) and anti-CD4 monoclonal antibodies (Prinz *et al*, 1991) have been shown to be beneficial in psoriasis treatment.

Several studies have suggested a key role for fibroblasts in initiating the epidermal abnormalities observed in psoriatic lesions (Saiag *et al*, 1985; Krueger and Jorgensen, 1990). In experimental models of the skin, fibroblasts from skin with and without lesions from psoriasis patients induce keratinocyte outgrowth by producing a soluble signal (Krueger and Jorgensen, 1990).

We found that cAMP-dependent protein kinase activity is low in fibroblasts and erythrocytes from psoriasis patients (Evain-Brion *et al*, 1986; Raynaud *et al*, 1989). This low level of activity of protein kinase, the intracellular mediator of cAMP action, was present in fibroblasts from both affected and unaffected skin. Oxidative changes to the enzymes impairs the binding of cAMP to the regulatory subunits in psoriatic cells (Raynaud *et al*, 1997). We have also shown that superoxide dismutase (SOD) activity, particularly that of the mitochondrial enzyme Mn-SOD, is significantly higher in fibroblasts from psoriasis patients than in normal subjects (Thérond *et al*, 1996). SOD activity acts as an antioxidant in cells, converting the superoxide ion $O_2^{\cdot-}$ into H_2O_2. Our data suggest that both $O_2^{\cdot-}$ and H_2O_2 levels are significantly higher in cultured psoriatic fibroblasts. The level of psoriatic fibroblast SOD activity is higher both in skin with and in skin without lesions (Thérond *et al*, 1996).

Protein oxidation has been shown to correlate with increased levels of carbonyls found in cellular macromolecules (Oliver *et al*,

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Abbreviations: DAPI, 4′,6-diamidono-2-phenylindole; DNP, dinitrophenylhydrazones; DNPH, 2,4-dinitrophenylhydrazine; INT, 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride; SOD, superoxide dismutase.

1987; Stadtman, 1992). The formation of reactive oxygen species under oxidative stress results in the carbonylation of proteins (Davis *et al*, 1987). Reactive oxygen species can produce carbonyl residues directly by oxidative cleavage of proteins or by the oxidation of arginine, lysine, threonine, and proline residues (Levine, 1983; Davis *et al*, 1987; Amici *et al*, 1989; Stadtman and Berlett, 1991). Carbonyl groups may also be introduced into proteins by reaction with α,β-unsaturated carbonyl fragments of membrane lipids during lipid peroxidation or by glycation and glycoxidation reactions (Berlett and Stadtman, 1997). Carbonyl residues can be readily detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) giving dinitrophenylhydrazones (DNP). The determination of hydrazones can, in turn, be used as a measure of the oxidative state of cells and tissues (Levine *et al*, 1990; Levine *et al*, 1994; Smith *et al*, 1996).

In this study, we used an indirect immunologic method to detect carbonyl residues in cultured fibroblasts from psoriasis patients. We used immunoblotting to demonstrate that there was more oxidative damage to the proteins in fibroblasts from affected and unaffected skin from psoriasis patients than in normal fibroblasts. Immunocytochemical techniques were used to assess intracellular oxidative damage in cultured fibroblasts and to show a specific increase *in situ* in the number of carbonyl residues in the dermis with and without lesions in sections from skin biopsies. This suggests that the changes in psoriatic fibroblasts are independent of the inflammatory lymphocyte infiltration observed in the tissue of lesions.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Seromed (Poly Labo Paul Block, Paris, France). Streptomycin and penicillin were obtained from Biological Industries (Israel). Trypsin and collagenase were supplied by Sigma-Aldrich Chimie SARL (Saint-Quentin Fallavier, France). All reagents used to study protein carbonylation, including DNPH solution, derivatization control solution, neutralization solution, rabbit anti-DNP antibody, and goat antirabbit antibody, were provided with the oxidized protein detection kit (Oxyblot, Appligene Oncor, Illkirch, France). Texas-Red-labeled goat antirabbit antibody and fluorescein-labeled goat antirabbit antibody were obtained from Jackson ImmunoResearch (Interchim, Asnières, France). All other chemicals were of analytical grade.

Tissue samples Samples of buttock and abdominal skin were obtained by biopsy from untreated adults with psoriasis (*psoriasis vulgaris* in flare-up for approximately 3 mo, 23–65 y of age) and from healthy age-matched control subjects. Psoriatic skin with and without lesions (tissue without lesions at a distance of at least 5 cm from the nearest lesion) was sampled. All subjects were Caucasian. This study was approved by the ethics committee of Hôpital Cochin.

Fibroblast culture Fibroblasts were isolated by enzymatic digestion of small pieces (4 mm) of dermis and were cultured as previously described (Thérond *et al*, 1996) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a humidified atmosphere of 5% CO_2/95% air at 37°C. Cells were used between the fourth and ninth passages.

Immunodetection of carbonylated proteins Subconfluent fibroblasts were washed twice with cold phosphate-buffered saline (PBS) and harvested by scraping into ice-cold buffer [0.25 M sucrose, 20 mM Tris(hydroxymethyl)-aminomethane (pH 7.4), 1 mM $MgCl_2$, 1 μg antipain per ml, and 1 μg leupeptin per ml]. The cells were recovered by centrifugation at $1000 \times g$ for 5 min. The cell pellet was homogenized using a Dounce homogenizer. Protein (10 μg determined by the Bradford method) was subjected to DNPH derivatization as described elsewhere (Levine *et al*, 1990, 1994; Nakamura and Goto, 1996; Smith *et al*, 1996). Protein (10 μg) was incubated with a control solution lacking DNPH as a control. The DNP-derivatized protein samples and controls were frozen for 18 h and were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gels. The separated proteins were Western blotted onto 0.45 μm pore size nitrocellulose membranes. The membranes were incubated with a rabbit anti-DNP antibody in PBS containing 0.05% Tween-20 and 1% bovine serum albumin (BSA) (1:150) for 1 h at room temperature, and then with a peroxidase-coupled goat antirabbit IgG antibody (1:300) for 1 h at room temperature. The membranes were then treated with chemiluminescence reagents (ECL, Amersham, France). Protein carbonylation was determined by densitometry of the bands on the autoradiograph. There was 28% intra-assay variation.

Immunocytochemistry Subconfluent fibroblasts cultured on glass slides were washed with PBS and fixed in 60% methanol, 40% acetone at –20°C for 8 min. The cells were washed six times with PBS, and were incubated with 1 × DNPH or with the derivatization control solution (1×) in PBS for 30 min at room temperature. Cells were washed 10 times with PBS and incubated in PBS with anti-DNP antibody (1:50) for 2 h at room temperature. The cells were washed six times with PBS, and were then incubated in PBS for 1 h with antirabbit IgG antibody coupled to Texas-Red or with antirabbit IgG antibody coupled to fluorescein (1:500). The cells were washed again, fixed in absolute ethanol for 1 min, covered with mounting medium containing 4′,6-diamidono-2-phenylindole (DAPI) (Vectashield, Vector Laboratories, Burlingame, CA), and examined with a fluorescence microscope under epifluorescent illumination with excitation-emission filters for rhodamine and DAPI.

Laser scanning cytometry The intensity of fluorescence was determined as previously described (Germain *et al*, 1993), using an interactive laser cytometer ACAS 570 (Meridian, Okemos, MI) equipped with a 5 W argon ion laser (Coherent, Palo Alto, CA). Fluorescence was assessed on a cell by cell basis, with 150 cells analyzed for each culture.

Immunohistochemistry Skin biopsy samples were rapidly frozen in isopentane cooled in liquid nitrogen, and stored at –80°C. Tissue sections (6 μm) were cut with a cryostat at –25°C and were stored at –80°C. Frozen sections from normal skin and from psoriatic skin with or without lesions were fixed in 60% methanol, 40% acetone at –20°C for 10 min. Tissue sections were sequentially rehydrated with PBS and washed five times, for 2 min each, in PBS. They were incubated for 1 h with DNPH solution (1×) or control derivatization solution (1×) as previously described (Nakamura and Goto, 1996). The sections were washed 10 times for 2 min each with PBS, and incubated with rabbit anti-DNP antibody in PBS (1:50) for 3 h. They were then washed with PBS for 20 min, and incubated with Texas-Red-labeled goat antirabbit IgG antibody (1:500) for 1 h in PBS. The sections were washed with PBS for 20 min, covered with mounting medium containing DAPI, and examined with a fluorescence microscope (Olympus, Rungis, France) under epifluorescent illumination with excitation-emission filters for rhodamine and DAPI.

Tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) assays with conditioned media from normal and psoriatic fibroblasts The cells were split and cultured for 2 d in Dulbecco's modified Eagle's medium with 10% serum. The cells were rinsed with 5 ml of PBS and then transferred to serum-free medium.

Conditioned serum-free medium was prepared from primary cultures of fibroblasts (normal, psoriatic fibroblasts obtained from skin with (IPS) and without (UIPS) lesion) by incubation for 24 h. Conditioned media were collected and immediately frozen (–20°C). The cells were counted (Counter Coulter, Beckman Coulter, Margency, France). The amount of TNF-α and IL-1β present in the 24 h supernatant was determined by enzymoimmunoassay (Immunotech, Luminy, France) according to the manufacturer's recommendation.

SOD activity As we previously described (Thérond *et al*, 1996), xanthine-xanthine oxidase was used to generate an $O_2^{\cdot-}$ flux and the reduction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to red formazan by $O_2^{\cdot-}$ was followed at 505 nm at 30°C. Each assay tube contained 50 mM 3-[cyclohexylamino]-1-propane-sulfonic acid buffer, pH 10.2, for determination of Cu,Zn SOD activity or 50 mM phosphate buffer, pH 7.8, for determination of total SOD activity, along with 1 mM ethylene diamine tetraacetic acid, 25 mM INT, 50 mM xanthine, 1 U catalase per ml, 0.05 mM bathocuproin disulfonate disodium salt, 0.13 mg BSA per ml, and enough xanthine oxidase to achieve the required 100% of noninhibition. Bathocuproin disulfonate disodium salt and BSA were added to inhibit nonenzymatic scavenging of $O_2^{\cdot-}$.

Mn-SOD activity was determined by adding cyanide (2 mM) to the assay mixture at pH 7.8 to inhibit Cu,Zn SOD activity, or was determined by subtracting the Cu,Zn SOD activity determined at pH 10.2 from the total SOD activity measured at pH 7.8. The two methods gave similar values. All data are expressed in units of SOD activity per mg of protein for fibroblasts.

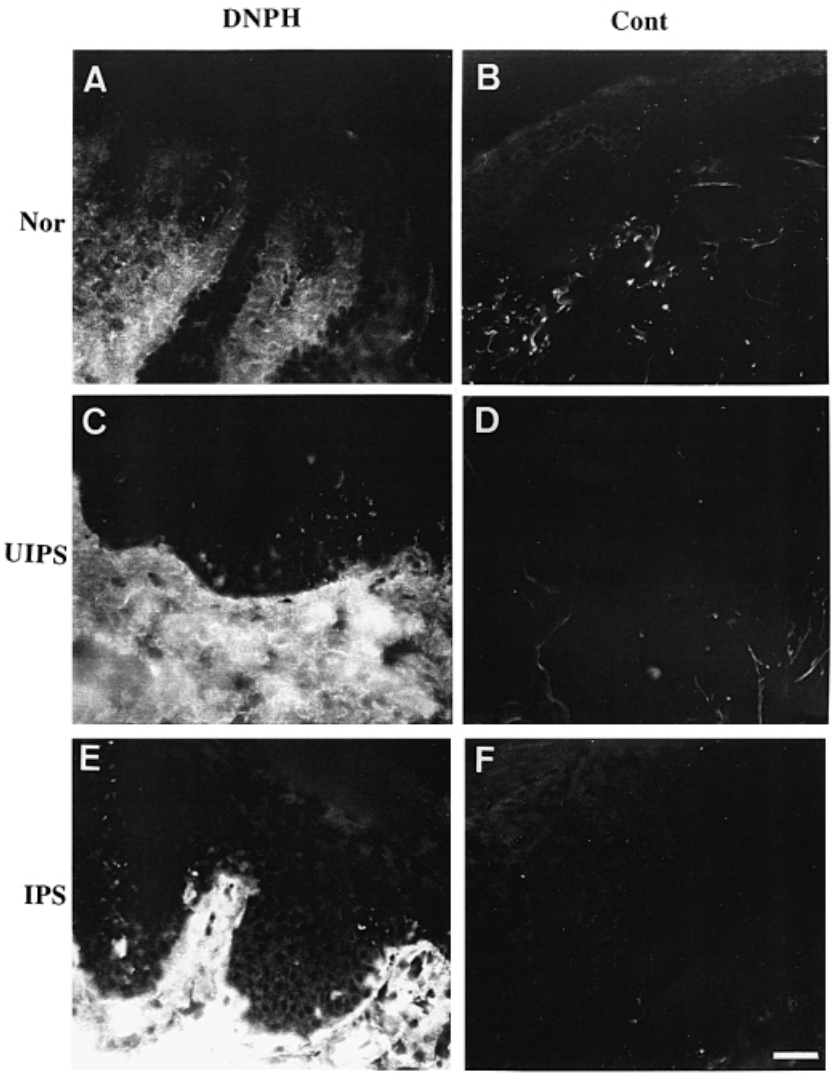

Figure 1. Oxidative damage to the dermis of normal and psoriatic skin. Immunohistochemical identification of the location of biomacromolecule-bound carbonyls in skin sections prepared from the biopsy samples of normal and psoriasis patients. Frozen sections (6 μm) of normal human skin (*A*, *B*), of psoriatic skin without lesions (*C*, *D*), and of psoriatic skin with lesions (*E*, *F*) were incubated with either DNPH solution (*A*, *C*, and *E*) or with control derivatization solution (*B*, *D*, and *F*) after fixation at –20°C in 60% methanol, 40% acetone. The derivatized skin sections were incubated with polyclonal rabbit anti-DNP antibody and then with a Texas-Red-linked antirabbit antibody. Skin sections were examined with a fluorescence microscope under epifluorescent illumination with excitation-emission filters for rhodamine (*scale bar*: 50 μm).

Statistical analysis The results are means ± SD or means ± SEM. The differences between the means for normal fibroblasts and psoriatic fibroblasts from skin with and without lesions were analyzed by the Mann–Whitney U test using Stat View Software (Abacus Concepts, Berkeley, CA). Values of $p < 0.05$ were considered to be significant. The Pearson correlation test was used for correlation analysis, and Student's t test using the SAS system software (SAS Institute, Cary, NC) was used to compare means. Spearman's rank correlation test was used for regression analysis.

RESULTS

Oxidative damage to the dermis of normal and psoriatic skin We first determined the extent of carbonylation using an *in situ*. immunohistochemical technique with DNPH labeling linked to an antibody system. Sections of skin from biopsies from three normal subjects and four subjects with psoriasis (skin with and without lesions) were studied (**Fig 1**). The dermis was intensely stained in both normal skin and psoriatic abdominal skin with and without lesions. No significant carbonylation was detected in the epidermis (**Fig 1*A***, ***C*** and ***E***). The dermis of sections prepared from psoriatic skin with and without lesions consistently showed higher levels of staining than the dermis of sections prepared from normal skin. There was little difference in the intensity of staining of the dermis of sections of skin with and without lesions taken from the same psoriatic patient (**Fig 1*C*** and ***E***). There was some weak, nonspecific immunostaining along elastic fibers, however, in both normal skin and psoriatic skin with and without lesions (**Fig 1*B***, ***D***, and ***F***).

Determination of carbonyl residues in cell extracts prepared from cultured fibroblasts We determined the extent of protein carbonylation in cell extracts from cultured fibroblasts. We checked that prooxidants increased the amount of carbonylated proteins by treating normal fibroblasts with 50 μM H_2O_2 for 4 h (**Fig 2**). An increase in the carbonylation of proteins was observed (1.43 arbitrary units for the control, 4.43 arbitrary units for the H_2O_2 treatment).

We then compared the extent of protein carbonylation in cell extracts of cultured fibroblasts prepared from biopsy samples from the skin of normal subjects and from lesional and nonlesional skin of psoriatic patients. Cell extracts were subjected to SDS electrophoresis and immunoblotted using an anti-DNP antibody (**Fig 3*A***). The same number of carbonylated protein bands was detected in normal and psoriatic fibroblasts, but the carbonylated protein bands were more intense in psoriatic fibroblasts. **Figure 3**(***B***), shows overall results of densitometry for all carbonylated protein bands present in each gel (*lane 2*) after the subtraction of nonspecific labeling in the corresponding control (*lane 1*) for each sample. The results are in arbitrary units and were obtained from cells prepared from 10 normal subjects or 12 psoriasis patients. The degree of carbonylation of proteins in fibroblast extracts from psoriatic skin without lesions (7.78 ± 3.10 arbitrary units; $p < 0.002$, mean ± SD) and from psoriatic skin with lesions (10.24 ± 4.45 arbitrary units; $p < 0.0006$, mean ± SD) was significantly higher than that of normal fibroblasts (3.69 ± 1.627 arbitrary units, mean ± SD) (**Fig 3*B***).

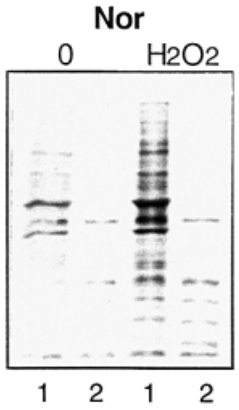

Figure 2. H_2O_2 prooxidant treatment increased carbonylated protein of normal fibroblasts. Immunoblot analysis of carbonyl residues of normal fibroblasts treated with 50 μM H_2O_2 for 4 h. Protein (10 μg) from normal fibroblasts (Nor) with or without H_2O_2 treatment were subjected to derivatization (*lane 1*) as described in *Materials and Methods*. *Lane 2* is the derivatization control. Proteins were subjected to SDS-PAGE in 10% gels and were immunoblotted with an anti-DNP antibody. Immune complexes were detected by chemiluminescence.

Intracellular location of oxidative damage in cultured fibroblasts Immunocytochemical techniques were used to determine the location of biomacromolecule-bound carbonyl groups in cultured fibroblasts prepared from normal skin (**Fig 4*A***, ***B***) and from psoriatic skin without (**Fig 4*C***, ***D***) and with (**Fig 4*E***, ***F***) lesions. DNP staining was diffuse throughout the cytoplasm, with no specific staining of the nucleus in all three types of cultured fibroblasts (**Fig 4*A***, ***C***, and ***E***). Incubation with control derivatization solution resulted in much fainter diffuse staining that was due to nonspecific labeling (**Fig 4*B***, ***D***, and ***F***).

DNP staining in normal and psoriatic fibroblasts from skin with and without lesions was quantified by laser scanning cytometry (**Fig 5*A***, ***B***, and ***C***). Normal fibroblasts showed significantly ($p < 0.0001$) less DNP staining (mean fluorescence intensity by cell ± SD, 2.42 ± 2.03 arbitrary units, $n = 100$) than psoriatic fibroblasts from nonlesional tissue (3.87 ± 2.59 arbitrary units, $n = 100$) and psoriatic fibroblasts from lesional tissue (3.67 ± 2.28 arbitrary units, $n = 100$). There was no significant difference in the intensity of DNP staining for psoriatic fibroblasts from skin with and without lesions taken from the same patient (**Fig 5*B***, ***C***). All three types of cell were stained to a similar extent when treated with control derivatization solution.

Production of TNF-α and Il-1β is significantly higher in psoriatic fibroblasts We have previously shown that Mn-SOD activity is higher in psoriatic fibroblasts than in normal fibroblasts (Thérond *et al*, 1996). Such an increase in Mn-SOD activity was also observed in this study (mean ± SEM, in lesional psoriatic fibroblasts IPS 400.86 ± 32.32 mU protein per mg; in nonlesional psoriatic fibroblasts UIPS 398.57 ± 39.65 mU protein per mg; in normal fibroblasts Nor 89.2 ± 9.51 mU protein per mg).

This increase in Mn-SOD activity in psoriatic fibroblasts may be due to an effect of inflammatory cytokines via an autocrine process. We therefore measured the concentrations of two cytokines known to induce an increase in Mn-SOD activity in cultured cells, TNF-α and Il-1β (Wong and Goeddel, 1988).

Both normal and psoriatic fibroblasts produced TNF-α and Il-1β. The concentrations of TNF-α and Il-1β were higher in the supernatant from psoriatic fibroblasts obtained from skin with [IPS, t test, $p = 0.1$ (TNF-α), $p = 0.1$ (Il-1β)] and without [UIPS, t test, $p = 0.0368$ (TNF-α), $p = 0.0153$ (Il-1β)] lesions than in the supernatant from normal fibroblasts [mean ± SEM, UIPS 249.2 ± 49.30 (TNF-α), IPS 158.0 ± 34.39 (TNF-α), Nor 97.33 ± 3.61 (TNF-α), UIPS 712.2 ± 138.5 (Il-1β), IPS 949.2 ± 211.89 (Il-1β), Nor 422 ± 34.4 (Il-1β)] (**Fig 6**).

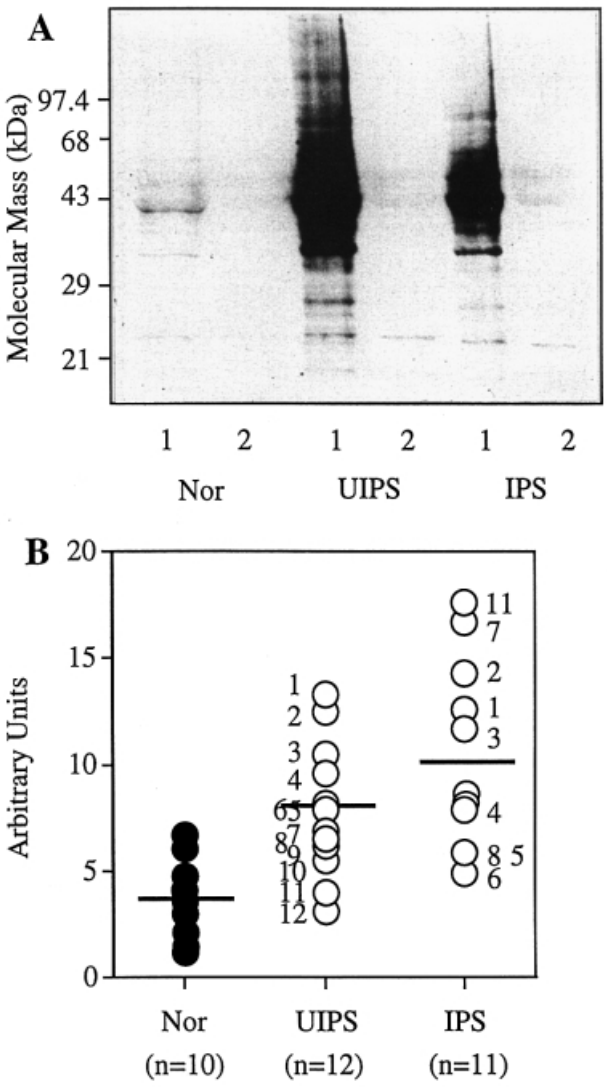

Figure 3. Increased carbonyl residues of proteins in cell extracts prepared from psoriatic versus normal fibroblasts. Immunoblot analysis of carbonyl residues in protein from normal and psoriatic fibroblasts. Proteins (10 μg) from normal fibroblasts (Nor) and from psoriatic fibroblasts prepared from uninvolved skin (UIPS) and from involved skin (IPS) were subjected to derivatization (*lane 1*) as described in *Materials and Methods*. *Lane 2* is the derivatization control. Proteins were subjected to SDS-PAGE in 10% gels and were immunoblotted with an anti-DNP antibody. Immune complexes were detected by chemiluminescence. Experiments were performed with several primary cultures. (*A*) Immunoblot obtained with one normal fibroblast culture and one psoriatic fibroblast culture. (*B*) Scanning densitometry of total carbonylated proteins from various fibroblast cultures prepared from 10 normal subjects and 12 subjects with psoriasis. Cultured fibroblasts prepared from skin with and without lesions from the same psoriasis patient are listed with the same number. Horizontal bars indicate the median value.

DISCUSSION

This study shows that the fibroblasts of psoriatic patients exhibit higher levels of oxidative damage as shown by the high levels of carbonylation *in vivo* and *in vitro*. The level of carbonylation was found to be higher in both fibroblasts and tissue sections prepared from skin biopsy samples of lesional and nonlesional tissue samples from psoriatic patients. This is consistent with our previous work showing that similar oxidative changes in cAMP-binding to the RI and RII regulatory subunits of cAMP-dependent protein kinase occur in affected and unaffected skin (Evain-Brion *et al*, 1986; Raynaud *et al*, 1989, 1997). Thus, the changes in psoriatic

fibroblasts may be independent of inflammatory lymphocyte infiltration, which only occurs in tissue with lesions (Christophers and Mrowietz, 1995).

Previous studies have shown that normal fibroblasts do show low levels of carbonylation (Oliver *et al*, 1987). High levels of carbonylation have been suggested to increase the susceptibility of these oxidized proteins to proteolysis (Davies, 1993; Dean *et al*, 1997). The levels of DNPH-reactive proteins do not change significantly in fibroblasts cultured from normal individuals until after the age of 60 (Oliver *et al*, 1987). The level of carbonylated proteins has also been shown to be independent of the duration of cell culture *in vitro* during early and intermediate passages (Oliver *et al*, 1987). Therefore, we used immunologic methods to detect carbonylated proteins in early and intermediate passages of cultured fibroblasts prepared from age-matched normal subjects and psoriasis patients below the age of 60 y, to negate any possible age-dependent increase in the level of DNPH-reactive protein. One of our psoriatic patients was 63 y old. The level of carbonylated protein detected in cultured fibroblasts from this patient, however, was at the lower end of the range observed for psoriatic fibroblasts.

The total amount of carbonylated protein correlated well with Mn-SOD activity in cells from psoriatic skin without lesions ($r^2 = 0.744$, $p < 0.05$). We have previously shown that mitochondrial Mn-SOD synthesis and activity is much higher in psoriatic than in normal fibroblasts (Thérond *et al*, 1996). This, along with the correlation between carbonylation and Mn-SOD activity, suggests that oxidative stress may lead to the overproduction of carbonyl proteins via the formation of H_2O_2, which then reacts with ferrous ions to form hydroxyl radicals. A similar dysfunction may be involved in the oxidative process leading to the deposition of aggregated amyloid β protein in Alzheimer's disease (Smith *et al*, 1992; Dykens, 1994).

Carbonyl residues were detected in the cytoplasm, but not in the nuclei, of cultured fibroblasts. Carbonyl residues were also specifically detected in the dermis in both normal and psoriatic skin sections. No carbonyl residues were detected in the epidermis, and particularly not in keratinocytes.

The observed changes in the levels of carbonylation may result either from increased generation of oxygen radicals or from changes in antioxidant activities. For example, fibroblasts and keratinocytes have been reported to have different antioxidant activities (Yonn *et al*, 1991; Shindo *et al*, 1994). Human epidermis has higher levels of antioxidant activities than does the dermis (Shindo *et al*, 1994).

Our results suggest that there is an increase in oxidative stress in psoriatic fibroblasts in skin both with and without lesions. Indeed, it has been suggested that fibroblasts may form a key element of an

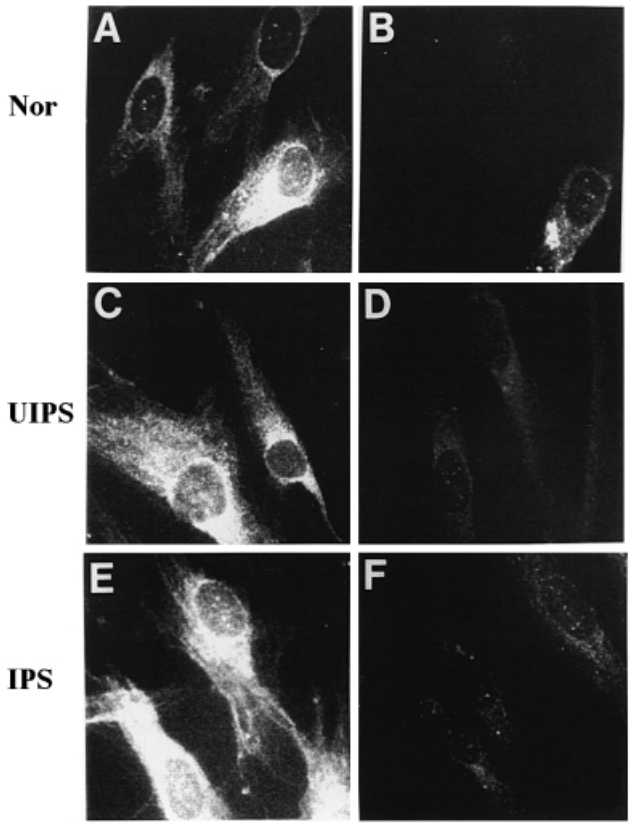

Figure 4. Cytosolic location of carbonyl residues in cultured fibroblasts. Detection of biomacromolecule-bound carbonyl residues in normal and psoriatic fibroblasts. Normal fibroblasts (*A*, *B*), psoriatic fibroblasts from uninvolved skin (*C*, *D*), and psoriatic fibroblasts from involved skin (*E*, *F*) were cultured on glass slides for 3 d. The cells were then fixed at –20°C in 60% methanol, 40% acetone. The fixed cells were incubated with either DNPH solution (*A*, *C*, and *E*) or with control derivatization solution (*B*, *D*, and *F*). The derivatized cells were incubated with polyclonal rabbit anti-DNP antibody and then with Texas-Red-linked antirabbit IgG antibody. Fibroblasts were examined with a fluorescence microscope under epifluorescent illumination with excitation-emission filters for rhodamine (*scale bar*: 10 μm).

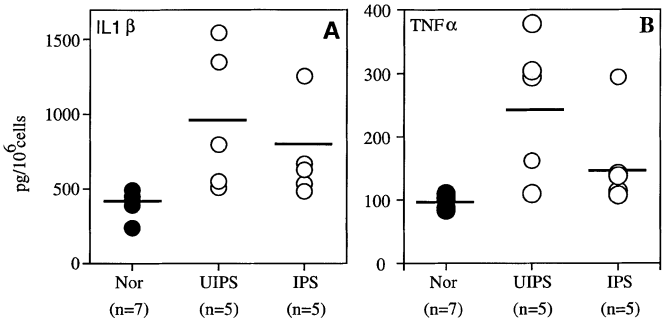

Figure 6. Increased production of TNF-α and IL-1β by psoriatic fibroblasts. TNF-α and IL-1β in 24 h conditioned serum-free media from normal fibroblasts (Nor) and from psoriatic fibroblasts from involved (IPS) and uninvolved (UIPS) skin, determined by enzyme immunoassay.

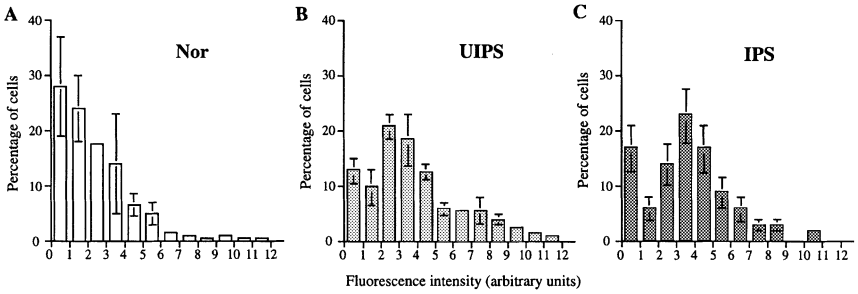

Figure 5. Analysis of fibroblast DNP staining by laser scanning cytometry. The immunocytochemical procedure was the same as that described in **Fig 2**, except that the second antibody used was a fluorescein-linked antirabbit IgG antibody. The fluorescence intensity of 150 cells from three normal (*A*), three psoriatic (skin without lesion) (*B*), and three psoriatic (skin with lesions) (*C*) fibroblast cultures was quantitated by laser scanning cytometry. Results are the mean fluorescence.

early warning system in tissues by producing chemokines in response to foreign intrusion (Smith *et al*, 1997). Psoriatic fibroblasts produce several cytokines, including TNF-α and IL-8 (Vassalli, 1992; Piguet, 1993; Konstantinova *et al*, 1996). Human fibroblasts in culture have been shown to release reactive oxygen species in response to stimulation with cytokines such as TNF-α and IL-β (Meier *et al*, 1989). Some of these secreted cytokines may act as autocrine inflammatory factors against psoriatic fibroblasts both *in situ* and in culture. The level of carbonylated proteins in psoriatic fibroblasts was correlated with the production of cytokines such as IL-1β only in fibroblasts obtained from skin without (UIPS) lesions (Pearson correlation coefficient, UIPS, 0.93439, $p = 0.02$, for IL-1β). Consistent with this, we found a correlation between the levels of a cytokine known to increase the oxidation in cells (IL-1β) and the levels of oxidative damage, as indicated by carbonylated protein levels. This study demonstrates essentially that the level of carbonylated proteins is higher in psoriasis. Other biomolecules, such as lipids, sugars, and DNA, may be also oxidized, as suggested by the immunocytochemistry and immunohistochemistry studies. Further investigation is required to evaluate such oxidative damage.

In conclusion, this study shows that, even before the formation of characteristic psoriatic lesions, fibroblasts in the lesion-free skin of psoriasis patients show signs of increased oxidative damage. This suggests that fibroblasts in the skin of psoriatic patients undergo changes even before psoriatic lesions are formed, and thus may be involved in the abnormal immune reactions leading to the onset of the disease. This is consistent with the suggestion of Smith *et al* (1997) that fibroblasts may be sentinel cells, part of the immune system, playing a key role in the cross-talk between hematopoietic circulating cells and skin-specific cells, such as keratinocytes.

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REFERENCES

Amici A, Levine R, Tsai L, Stadtman ER: Conversion of amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation. *J Biol Chem* 264:3341–3346, 1989

Bato-Csorgo Z, Hammerberg C, Voorhees JJ, Cooper KD: Intralesional T-lymphocyte activation as a mediator of psoriatic epidermal hyperplasia. *J Invest Dermatol* 105:89S–94S, 1995

Berlett BS, Stadtman ER: Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 272:20313–20316, 1997

Christophers E: The immunopathology of psoriasis. *Int Arch Allergy Immunol* 110:199–206, 1996

Christophers E, Mrowietz U: The inflammatory infiltrate in psoriasis. *Clin Dermatol* 13:131–135, 1995

Christophers E, Sterry W: Psoriasis. In: Fitzpatrick TB, Eisen AZ, Wolf K, Freedberg JM, Austen KF (eds). *Dermatology in General Medicine*. New York: McGraw-Hill, 1993

Davies KJ: Protein modifications by oxidants and the role of proteolytic enzymes. *Biochem Soc Trans* 21:346–353, 1993

Davis KJA, Delsignore ME, Lin SW: Protein damage and degradation by oxygen radicals II. Modification of amino acids. *J Biol Chem* 262:9902–9907, 1987

Dean RT, Fu S, Stocker R, Davies HJ: Biochemistry and pathology of radical-mediated protein oxidation. *Biochem J* 324:1–8, 1997

Dykens JA: Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{++} and Na^{+}: implications for neurodegeneration. *J Neurochem* 63:584–591, 1994

Elder JT: Cytokine and genetic regulation of psoriasis. *Adv Dermatol* 10:99–133, 1995

Elder JT, Nair RP, Guo SW, Henseler T, Christophers E, Voorhees JJ: The genetics of psoriasis. *Arch Dermatol* 130:216–224, 1994

Ellis CN, Fradin MS, Messana J: Cyclosporin for plaque-type psoriasis, results of a multidose, double-blind trial. *N Engl J Med* 324:277–284, 1991

Evain-Brion D, Raynaud F, Laurent P, Plet A, Leduc B: Deficient cyclic AMP-dependent protein kinases in human psoriatic cells. *Proc Natl Acad Sci USA* 83:5272–5276, 1986

Germain PS, Metezeau P, Tiefenauer LX, Kiefer H, Ratinaud MH, Habrioux G: Use of a biotinyl-estradiol derivative to demonstrate estradiol-membrane binding sites on adherent human breast cancer MCF-7 cells. *Anticancer Res* 13:2347–2354, 1993

Gottlieb SL, Gilleaudeau P, Johnson R, Estes L, Woodworth TG, Gottlieb AB, Krueger JG: Response of psoriasis to a lymphocyte-selective toxin (DAB_{389}IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. *Nature Med* 1:442–447, 1995

Henseler T: The genetics of psoriasis. *J Am Acad Dermatol* 37:S1–S11, 1997

Konstantinova NV, Duong DM, Remenyik E, Hazarika P, Chuang A, Duvic M: Interleukin-8 is induced in skin equivalents and is highest in those derived from psoriatic fibroblasts. *J Invest Dermatol* 107:615–621, 1996

Krueger GG, Duvic M: Epidemiology of psoriasis: clinical issues. *J Invest Dermatol* 102:14S–18S, 1994

Krueger GG, Jorgensen CM: Experimental models for psoriasis. *J Invest Dermatol* 95:56S–58S, 1990

Levine RL: Oxidative modification of glutamine synthetase II. Characterization of the ascorbate system. *J Biol Chem* 258:11828–11833, 1983

Levine RL, Garland D, Oliver CN, *et al*: Determination of carbonyl content in oxidatively modified proteins. *Meth Enzymol* 186:464–478, 1990

Levine RL, Williams JA, Stadtman ER, Shacter E: Carbonyl assays for determination of oxidatively modified proteins. *Meth Enzymol* 233:346–357, 1994

Meier B, Radeke HH, Selle S, Younes M, Sies H, Resch K, Habermehl GG: Human fibroblasts release reactive oxygen species in response to interleukine-1 or tumor necrosis factor-α. *Biochem J* 263:539–545, 1989

Nakamura A, Goto S: Analysis of protein carbonyls with 2,4-dinitrophenylhydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. *J Biochem* 119:768–774, 1996

Oliver CN, Ahn BW, Moerman EJ, Goldstein S, Stadtman ER: Age-related changes in oxidized proteins. *J Biol Chem* 262:5488–5491, 1987

Piguet PF: TNF and the pathology of the skin. *Res Immunol* 144:320–326, 1993

Prinz J, Braun-Falco O, Meurer M, Daddona P, Reiter C, Rieber P, Riethmuller G: Chimaeric CD4 monoclonal antibody in treatment of generalised pustular psoriasis. *Lancet* 338:320–321, 1991

Raynaud F, Gerbaud P, Enjolras O, Gorin I, Anderson WB, Evain-Brion D: A cAMP binding abnormality in psoriasis. *Lancet* 1:1153–1156, 1989

Raynaud F, Evain-Brion D, Gerbaud P, Marciano D, Gorin I, Liapi C, Anderson WB: Oxidative modulation of cyclic AMP-dependent protein kinase in human fibroblasts: possible role in psoriasis. *Free Radic Biol Med* 22:623–632, 1997

Saiag P, Coulomb B, Lebreton C, Bell E, Dubertret L: Psoriatic fibroblasts induce hyperproliferation of normal keratinocytes in a skin equivalent model *in vitro*. *Science* 230:669–672, 1985

Shindo Y, Witt E, Han D, Epstein W, Packer L: Enzymic and non-enzymic antioxidants in epidermis and dermis of human skin. *Soc Invest Dermatol* 102:122–124, 1994

Smith CD, Carney JM, Tatsuhot T, Statman ER, Floyd RA, Markesbery WR: Protein oxidation in aging brain. *Ann N Y Acad Sci* 663:100–119, 1992

Smith MA, Perry G, Richey PL, Sayre LM, Anderson VE, Beal MF, Kowall N: Oxidative damage in Alzheimer's. *Nature* 382:120–121, 1996

Smith RS, Smith TJ, Blieden TM, Phipps RP: Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. *Am J Pathol* 151:317–322, 1997

Stadtman ER: Protein oxidation and aging. *Science* 257:1220–1224, 1992

Stadtman ER, Berlett BS: Fenton chemistry. Amino acid oxidation. *J Biol Chem* 266:17201–17211, 1991

Thérond P, Gerbaud P, Dimon S, Anderson WB, Evain-Brion D, Raynaud F: Antioxidant enzymes in psoriatic fibroblasts and erythrocytes. *J Invest Dermatol* 106:1325–1328, 1996

Vassalli P: The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* 10:411–452, 1992

Wong GH, Goeddel DV: Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. *Science* 242:941–944, 1988

Yonn JJ, Norris DA, Yrastorza DG, Buno IJ, Leff JA, Hake SS, Repine JE: Disparate antioxidant enzyme activities in cultured human cutaneous fibroblasts, keratinocytes and melanocytes. *J Invest Dermatol* 97:405–409, 1991