Collagen of Chronically Inflamed Skin Is Over-Modified and Upregulates Secretion of Matrix Metalloproteinase 2 and Matrix-Degrading Enzymes by Endothelial Cells and Fibroblasts

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In order to investigate the properties of collagen in chronically inflamed tissue, we isolated collagen from the ear skin of mice with chronic contact dermatitis and examined its biochemical characteristics and the functions that regulate the secretion of matrix metalloproteinase 2 and collagen-degrading enzymes from endothelial cells and fibroblasts. Collagen in skin with chronic contact dermatitis comprised 60% type I collagen and 40% type III collagen, which latter is higher than the content of type III collagen in control skin (35%). The denaturation temperature was higher (42°C) than that of control skin (39°C). The α2 chain of type I collagen was over-hydroxylated at both proline and lysine residues. Segment-long-spacing crystallites of type I collagen were unusually connected in tandem. Collagen of chronically inflamed skin was less susceptible to matrix metalloproteinase 2 after heat denaturation. Endothelial cells and fibroblasts secreted an increased amount of matrix metalloproteinase 2 when cultured on a gel formed from the collagen of chronically inflamed skin. Collagen-degrading activity secreted from fibroblasts was also upregulated when cells were in contact with collagen of chronically inflamed skin. These results suggest that the collagen in chronically inflamed tissue has altered biochemical characteristics and functions, which may affect the pathogenesis of the chronic skin disease. ***Key words: denaturation temperature/2,4-dinitrofluorobenzene/hydroxylysine/hydroxyproline/segment-long-spacing crystallites. J Invest Dermatol 121:1317–1325, 2003***

Collagen is a major component of the extracellular matrix in connective tissues, serving not only as a structural component of connective tissues but also regulating the activity of cells through the interaction with cell surface receptors. Collagen is synthesized through multiple post-translational processing steps. Propeptides of procollagen are removed by specific enzymes. The proline residue is hydroxylated with 4-prolylhydroxylase or 3-prolylhydroxylase. The lysine residue is hydroxylated with lysine hydroxylase, and some of the hyroxylysine residues are glycosylated with galactose or glucosylgalactose. These post-translational modifications occur intracellularly prior to folding into the triple-helical conformation. Collagen is further modified extracellularly by lysyl oxidase, and deaminated lysine or hydroxylysine is involved in the formation of stable cross-links (Kucharz, 1992).

The post-translational modifications are essential steps for collagen to serve as a structural component with high tensile strength and as a substratum for cell adhesion. It has been reported that the mode of post-translational modifications changes in pathologic conditions. For example, cross-links of collagen are chemically altered in inflamed skin (Bailey *et al*, 1973). The amount of pyridinoline increases in skin fibrosis induced by infection with chromomycosis (Ricard-Blum *et al*, 1993). Synthesis of collagen by fibroblasts *in vitro* at an elevated temperature resulted in an increase of hydroxylysine and glycosylated hydroxylysine, and the diameter of the reconstituted fibril was smaller than normal (Torre-Blanco *et al*, 1992). In addition to the post-translational modifications during biosynthesis, collagen may be modified extracellularly by reaction with active oxygen (Ohshima *et al*, 1993) or sugars (Kawano *et al*, 1990) or by maturation in a serum-containing environment (Nishikawa *et al*, 1987). *In vitro* modified collagen exhibits the altered property that it serves as a substratum for fibroblasts (Kawano *et al*, 1990; Ohshima *et al*, 1993; Akutsu *et al*, 2000). Thus, it seems possible that the post-translationally over-modified collagen in diseased tissue has the altered ability to regulate the activity of cells. It remains unclear, however, whether collagen in diseased tissue actually acquires the ability to modulate the pathogenesis of the disease or its repair.

It has been recently reported that chronic inflammation is quite different from acute inflammation in the expression and distribution of extracellular matrix components and the activation of a matrix-degrading enzyme (Kusubata *et al*, 1999). In chronically

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Abbreviations: MMP, matrix metalloproteinase; SLS crystallites, segment-long-spacing crystallites.

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inflamed skin, the degradation of fibronectin and tenascin-C was suppressed, and the activity of active matrix metalloproteinase 2 (MMP-2) was increased compared with acute inflammation (Kusubata *et al*, 1999). In this study, to investigate whether chronic inflammation is associated with biochemical changes of collagen and whether the collagen in chronically inflamed tissue acquires the ability to modify the pathogenesis of chronic inflammation, we isolated collagen from skin with chronic contact dermatitis and investigated its biochemical properties, susceptibility to MMP, and the ability to regulate the secretion of matrix-degrading enzymes by endothelial cells and fibroblasts.

MATERIALS AND METHODS

Induction of chronic contact dermatitis Female BALB/cA mice were purchased from Japan Clea (Tokyo, Japan) and used between 7 and 8 wk of age. Chronic contact dermatitis was induced as reported previously (Kusubata *et al*, 1999). Briefly, 10 μL of 0.2% (vol/vol) 2,4-dinitrofluorobenzene (DNFB) in acetone was applied to both the outer and inner sides of the right ear every 3 d for 18 d. The left ear received vehicle alone. The experiments reported were approved by the Nippi Research Institute of the Biomatorix, Nippi Inc.

Extraction and purification of collagen Both ears were obtained on day 0 (normal ear) and day 18 (chronic phase of inflammation) (Kusubata *et al*, 1999). The ears were washed at 4°C for 4–5 d in phosphate-buffered saline (pH 7.6) containing 0.1 M ethylenediamine-N,N,N′,N′-tetraacetic acid, disodium salt, 0.1 mM N-ethylmaleimide, and 10 μM phenylmethylsulfonyl fluoride. Then, the ear was separated into the inner and outer side, and cartilage was removed from the skin. The skin was swelled with 1 mL of 0.5 M acetic acid at 4°C for 1 d. After adding another milliliter of 0.5 M acetic acid, the skin was homogenized in a glass homogenizer and left at 4°C for 1 d to extract acid-soluble collagen. After centrifugation at 24,100g (30 min, 4°C), 2 mL of 0.01% pepsin (wt/vol) in 0.5 M acetic acid was added to the precipitate and allowed to react at 4°C for 1 d to extract pepsin-solubilized collagen. After centrifugation at 24,100g (30 min, 4°C), the precipitate was stored at −20°C as the insoluble residue. Pepsin-solubilized collagen in supernatant was purified through salt fractionation. 4 M NaCl solution was added to the collagen solution under continuous stirring to obtain a final concentration of 1 M and left at 4°C for 1 d. The samples were centrifuged at 34,800g (30 min, 4°C), and the precipitate was dissolved in 10 times (vol/wt) Milli Q water. Collagen was precipitated by dialysis against 5 mM Tris−HCl buffer (pH 7.4). The precipitated collagen was then redissolved in a 10 times volume of 50 mM acetic acid at 4°C and dialyzed against 5 mM acetic acid (Chandra Rajan, 1978). The collagen solution of 1 mg per mL concentration was stored at −20°C until use. Extraction of type I collagen from the dorsal skin of the mouse was carried out using pepsin (Chandra Rajan, 1978). Bovine type III collagen was extracted from the fetal skin by pepsin (Chandra Rajan, 1978). The amount of collagen was calculated from the amount of hydroxyproline.

Electrophoresis Collagen was mixed with sodium dodecyl sulfate sample buffer (pH 6.8). Samples were neutralized with 1 M Tris−HCl buffer (pH 9.0) and heated at 100°C for 3 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 5% gel was carried out in either the presence or the absence of 4% 2-mercaptoethanol in the sample buffer. α1(III) chain of type III collagen was identified as a single band by electrophoresis in a reduced condition in the presence of 3.6 M urea (Hayashi and Nagai, 1979). Gels were stained with Coomassie Brilliant Blue, and the amount of each band was determined by densitometry. The content of type III collagen is expressed as

$$\{\alpha I(III)/[\alpha 1(I) + \alpha 2(I) + \alpha 1(III)]\} \times 100\ (\%)$$

Digestion with V8 protease and amino acid sequencing Collagen was digested with V8 protease in 0.1 M CH_3COONH_4 buffer (pH 4.0) at 37°C for 16–40 h at an enzyme−substrate ratio of 1/20–1/2. After being neutralized by adding 1 M Tris−HCl (pH 9.0), digested collagen was subjected to SDS-PAGE on 10% gel under nonreducing conditions. The gel was visualized by silver staining, or electrotransferred to an Immobilon-P^{SQ} transfer membrane (Millipore, Bedford, MA) and stained with Coomassie Brilliant Blue. Peptide bands were cut from the membrane and subjected to amino acid sequencing by 491 Protein Sequencer (Applied Biosystems, Foster, CA).

Amino acid analysis Collagen was neutralized with 1 M Tris−HCl buffer (pH 9.0) and heated at 60°C for 15 min under reducing conditions with 50 mM dithiothreitol. Each component of collagen was fractionated with a 40 min gradient of 2%–40% acetonitrile at 40°C by reverse-phase high performance liquid chromatography (Irika, Kyoto, Japan). Each fraction containing α2(I) chain of type I collagen was collected, dried, and hydrolyzed with 6 N HCl at 110°C for 24 h in the gas phase. Then, the reaction mixture was dissolved in 0.02 N HCl. Amino acid analysis was performed with L-8500 amino acid analyzer (Hitachi, Tokyo, Japan) using citrate buffer and sodium chloride gradient. Amino acid in the eluate was monitored by postcolumn reaction with ninhydrin.

Measurement of denaturation temperature Collagen diluted with 5 mM acetic acid at a concentration of 0.1 mg per mL was placed in a Jasco-600 spectropolarimeter (Japan Spectroscopic, Japan). Circular dichroism ellipticity of the collagen at 221 nm was monitored by increasing the temperature at a constant rate of 1/4°C per min from 20°C to 50°C. Denaturation temperature was defined as the temperature of the mid-point of ellipticity between 20°C and 50°C.

Electron microscopy of segment-long-spacing (SLS) crystallites In order to form SLS crystallites, pepsin-solubilized collagen, either purified by salt fractionation or without purification, was dialyzed at 4°C first against 0.5 M acetic acid for 2 d (pH 2.5–2.6) to dissociate acid–labile interactions, and then against 0.2% ATP2Na in 0.1 M acetic acid (pH 2.8–3.0) for 1 d. Samples were taken on collodion-coated grids, negatively stained with 2% ammonium molybdate, and examined with a 7100 Electron Microscope (Hitachi) (Kobayashi *et al*, 1985).

Digestion with MMP Collagen was neutralized by adding 1 M Tris−HCl (pH 9.0). Heat denaturation of the collagen was carried out by keeping it at 60°C for 15 min. Ten microliters of 1 mg per mL collagen, either native or heat-denatured, was subjected to digestion in the presence of 1 mM $CaCl_2$ with 20 μL of 0.5 unit per mL human MMP-1 (Wako, Japan) at 20°C for 6 d or at 35°C for 24 h. Collagen, either native or heat-denatured, was also digested with human MMP-2 or MMP-9 (Wako) by adding (1) 20 μL of 0.5 unit per mL solution at 37°C for 24 h, or (2) 10 μL of 0.5 unit per mL solution at 37°C for 16 h, or (3) 30 μL of 0.5 unit per mL solution at 37°C for 24 h or 42 h. The digested collagen was subjected to SDS-PAGE on 7.5% gel under nonreducing conditions and stained with Coomassie Brilliant Blue R250.

Cell culture Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Small pieces of the dorsal skin of the mouse were placed on a culture dish and maintained for 10 d. Adherent cells that outgrew from the skin were harvested by trypsin treatment and used as skin fibroblasts. Bovine carotid endothelial cells were kindly provided by Dr Kaoru Irie (Tokyo Women's Medical University). Collagen gel was prepared with pepsin-solubilized collagen of the ear. Two hundred microliters of 1 mg per mL collagen solution was mixed with 50 μL of serum-free 5 × DMEM and transferred into a 48-well plate (final concentration 0.8 mg per mL). The plate was incubated at 37°C for 2 h to allow collagen polymerization. Cells were trypsinized and washed twice in serum-free 1 × DMEM. Thereafter, cells suspended in serum-free 1 × DMEM (1.3×10^4 per well) were added on the gel and incubated at 37°C for 24 h. In order to examine effects of a ratio of type III collagen in a gel, normal skin-derived bovine type III collagen was mixed with mouse type I collagen at a ratio of 0%, 30%, or 50%, and gels were formed as described above.

Zymography Endothelial cells and fibroblasts were cultured in serum-free medium for 24 h on a reconstituted collagen gel, and the culture medium was centrifuged at 16,400g for 5 min. The supernatant was subjected to electrophoresis under nonreducing conditions in two SDS-PAGE 10% gels containing 3.2 mg per mL gelatin. The gels were washed twice in 2.5% Triton X-100 for 30 min each and equilibrated in 50 mM Tris−HCl (pH 7.4). Then, one gel was incubated for reaction of MMP-2 in 50 mM Tris−HCl containing 5 mM $CaCl_2$ and 200 mM NaCl at 37°C for 16 h. The other gel was incubated in the absence of calcium in 50 mM Tris−HCl containing 10 mM ethylenediamine-N,N,N′,N′-tetraacetic acid, disodium salt, and 200 mM NaCl at 37°C for 16 h. Both gels were stained with Coomassie Brilliant Blue. Densitometric analysis of the proteolytic band was performed with Bio-profile image analyzer (Vilber Lourmat, Marne-La-Vallee, France). The activity of MMP-2 was calibrated by measuring the MMP-2 activity in serially diluted culture supernatant, and relative activity was determined in the linear range of unsaturated amount of MMP-2 activity.

Collagenase assay Cells were cultured in serum-free medium for 24 h on a reconstituted collagen gel, and the culture medium was centrifuged at 16,400g for 5 min. Secreted collagenase activity was determined using fluorescein-isothiocyanate-labeled soluble bovine skin collagen as a substrate (Nagai *et al*, 1984). Fifty microliters of culture medium was mixed with 100 μL of substrate buffer (0.1 M Tris–HCl, pH 7.5/0.05% fluorescein-isothiocyanate-labeled collagen solution) and incubated at 35°C for 24 h. The reaction was stopped by adding 80 mM o-phenanthroline at 35°C for 10 min. The samples were cooled on ice, vigorously mixed with ethanol/Tris–HCl, pH 9.5 (7:3), and allowed to stand on ice for 15 min. After centrifugation at 7,300g for 10 min, the fluorescence change of the supernatant was recorded in a 96-well plate with excitation at 485 nm and emission at 530 nm using a CytoFluor Series 4000 (PerSeptive Biosystems, MS). One unit of collagenase activity is defined as the amount of enzyme degrading 1 μg of collagen per min under the conditions employed.

Transmission electron microscopy and fibril diameter measurement Collagen fibrils were prepared as described above in *Cell culture*. Fibrillar collagen was centrifuged at 16,400g rpm for 5 min for condensation and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 37°C for 4 h. After dehydration with a graded ethanol series, samples were embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultra-thin sections were stained with 0.5% uranyl acetate (pH 4.4) and lead citrate, and then examined with a 7100 Electron Microscope (Hitachi). Collagen fibrils were photographed at 100,000 ×, and the diameter of 600 fibrils was measured by NIH Image (version 1.61).

RESULTS

Biochemical changes of collagen in chronic inflammation In the normal skin of the ear, the amount of acid-soluble collagen and pepsin-solubilized collagen was 20% and 75% of the total collagen, respectively, with 5% insoluble collagen. Collagen in inflamed tissue was isolated from the ear with chronic contact dermatitis on day 18, when distribution of extracellular matrix components is quite different from that in normal skin or in skin under acute inflammation (Kusubata *et al*, 1999). No obvious change was observed either in the total amount of collagen per ear or in the ratio of acid-soluble collagen to pepsin-solubilized collagen on day 18 in DNFB-treated skin. The amount of type III collagen in pepsin-solubilized collagen was 35% in the normal skin and the vehicle-treated skin on day 18, and 40% in DNFB-treated skin on day 18. Mobility in SDS-PAGE of type I collagen and type III collagen in DNFB-treated skin was slightly but reproducibly smaller than in normal skin or vehicle-treated skin (**Fig 1**). This mobility change was observed both in nonreducing and reducing conditions. These results suggest the possibility that type I collagen and type III collagen in skin with chronic contact dermatitis is biochemically different from that in control skin. The amount of acid-soluble collagen was so small that some of the analyses were difficult to perform. Therefore, properties of pepsin-solubilized collagen were examined further in this study.

The denaturation temperature of collagen determined by circular dichroism is summarized in **Fig 2**. There was no clear difference between normal skin (39.0°C) and vehicle-treated skin (39.2°C). In contrast, the denaturation temperature of collagen in DNFB-treated skin (41.9°C) was evidently higher than that of controls. It was reported that denaturation temperature does not differ between type I and type III collagen (Byers *et al*, 1974). Therefore, these results suggest that the higher denaturation temperature cannot be attributed to the 5% increase in the content of type III collagen itself but rather derives from modifications of type I and/or type III collagen in DNFB-treated skin.

We then examined the sensitivity of collagen to V8 protease. **Figure 3** shows SDS-PAGE of V8-digested collagen peptides. Digestion of collagen of DNFB-treated skin resulted in an altered peptide pattern: a number of peptide bands increased in amount, whereas others decreased (**Fig 3**). Among these bands, amino acid sequencing of three bands was performed successfully. The amino terminus of an 84 kDa band, which

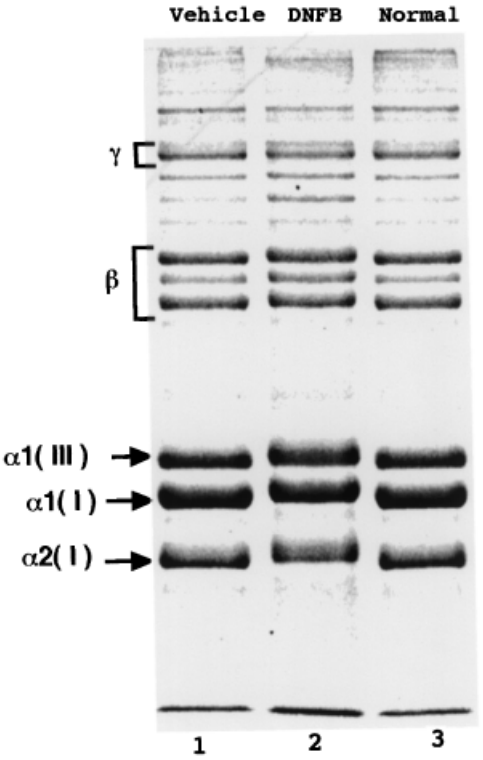

Figure 1. Collagen of chronically inflamed skin has a lower mobility in SDS-PAGE. Collagen extracted from vehicle-treated skin (*lane 1*), DNFB-treated skin (*lane 2*), and normal skin (*lane 3*) was subjected to SDS-PAGE in a reducing condition. β, β chain; γ, γ chain.

increased in amount, was Leu273 of α2(I). The amount of 83 kDa and 63 kDa bands decreased, and their amino termini were Val290 and Ala495 of α2(I), respectively. These results indicate that α2(I) in DNFB-treated skin has a different susceptibility to V8 protease. Inflammation and wound healing activate 4-prolylhydroxylase, which hydroxylates proline (Fullana *et al*, 1993). Therefore, the degree of hydroxylation of proline and lysine residues was examined. α2(I) was isolated by high performance liquid chromatography, but the peaks of α1(I) and α1(III) overlapped and could not be thus isolated. Therefore, α2(I) was subjected to amino acid analysis. The ratio of hydroxyproline and hydroxylysine of α2(I) in DNFB-treated skin was significantly higher than that of vehicle-treated skin or normal skin (**Table I**). These results indicate that α2(I) was over-hydroxylated at both the proline and lysine residues in chronically inflamed skin. We do not exclude the possibility that α1(I) and/or α1(III) is also over-hydroxylated because their mobility is decreased too as for α2(I) (**Fig 1**).

Type I collagen in chronically inflamed skin forms 0.4D-staggered SLS crystallites When SLS crystallites were made from purified collagen of normal, vehicle-treated, or DNFB-treated skin, they were scattered on a grid without a marked association between crystallites. This was also the case for unpurified collagen of control skin (**Fig 4*A***, ***C***). In contrast, when unpurified collagen of DNFB-treated skin was used, many of the crystallites were connected in tandem and overlapped at terminal 0.4*D* in length (*D* is the length of one cross-striation period of 67 nm) (**Fig 4*B***), giving a raft-like appearance at a lower magnification (**Fig 4*D***). A banding pattern of SLS crystallites is determined by the amino acid sequence of aggregated collagen molecules (Kobayashi *et al*, 1986). Analysis of the banding pattern of SLS crystallites indicated that they were made of type I collagen (Kobayashi

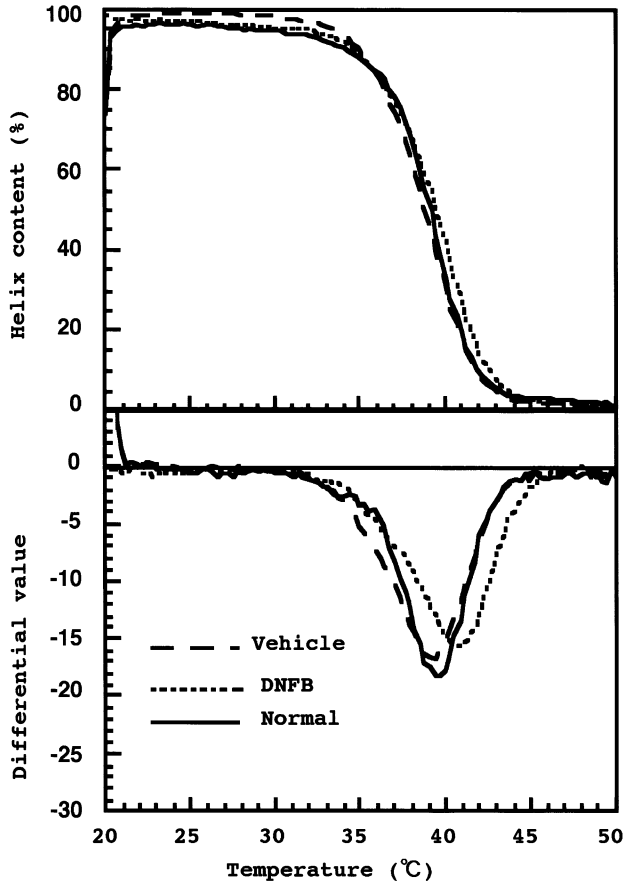

Figure 2. Collagen of chronically inflamed skin has a higher denaturation temperature. Melting temperature was measured by circular dichroism ellipticity (*upper figure*) and differential values are shown in the *lower figure.*

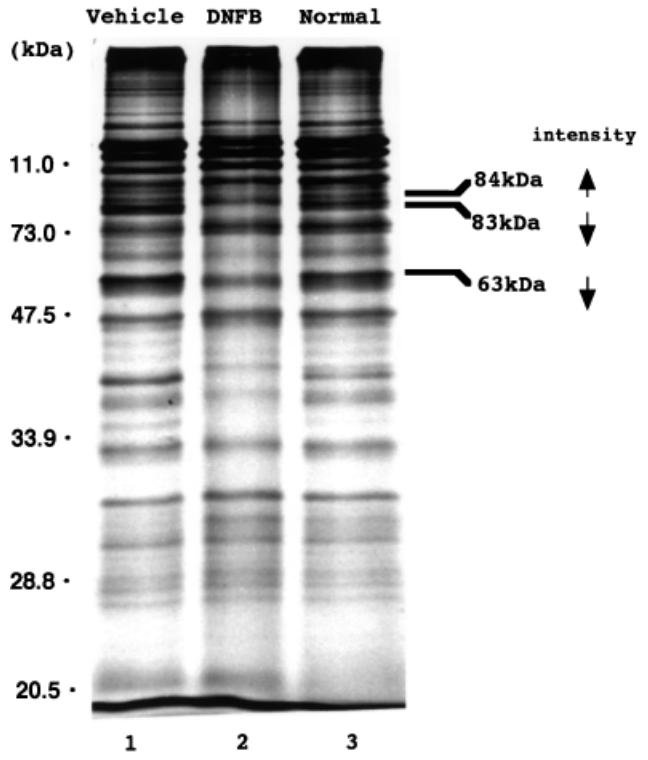

Figure 3. Collagen of DNFB-treated skin has altered susceptibility to V8 protease. SDS-PAGE of collagen in vehicle-treated skin (*lane 1*), DNFB-treated skin (*lane 2*), and normal skin (*lane 3*) after digestion with V8 protease. *Arrow* indicates an increase or decrease in band intensity of collagen of DNFB-treated skin.

Table I. Hydroxylation of proline and lysine in the α2 chain of type I collagen

	Vehicle	DNFB	Normal
Amino acid per 1000 residues			
Pro	108 ± 0.7	103 ± 2.8	109 ± 0.9
Hyp	81 ± 1.4	85 ± 2.7	82 ± 1.1
Pro + Hyp	189 ± 1.5	188 ± 1.7	192 ± 1.8
Lys	21 ± 0.6	19 ± 0.2	21 ± 0.6
Hyl	10 ± 0.8	12 ± 0.5	10 ± 0.1
Lys + Hyl	31 ± 1.3	31 ± 0.7	31 ± 0.7
Hydroxylation (%)			
Pro	42.8 ± 0.6	45.3 ± 1.4*	42.9 ± 0.4
Lys	33.1 ± 0.4	37.4 ± 0.4*	34.7 ± 0.7

Pro, proline; Hyp, hydroxyproline; Lys, lysine; Hyl, hydroxylysine.
Mean ± SD (n = 3).
*$p < 0.05$ in comparison with Vehicle and Normal.

et al, 1986), and the banding pattern did not differ among three groups (**Fig 4*A*, *B***). These results suggest the possibility that type I collagen of chronically inflamed skin is intermolecularly cross-linked in its terminal region more frequently than collagen of control skin, and that such cross-linked molecules may be removed by purification through salt fractionation.

Collagen of chronically inflamed skin forms thin fibrils *in vitro* The diameter of collagen fibrils reconstituted *in vitro* changes in association with post-translational modifications of collagen (Torre-Blanco *et al*, 1992). Therefore, we reconstituted collagen fibrils *in vitro* and their diameter was measured by electron microscopy. As shown in **Fig 5**, the diameter of collagen fibrils was significantly thinner for collagen in DNFB-treated skin (35.1 ± 6.1 nm) than in normal skin (48.0 ± 4.2 nm) or vehicle-treated skin (49.6 ± 4.8 nm). The effect of the content of type III collagen in a gel was examined by adding an increasing amount of normal skin-derived type III collagen to a reconstituted gel. When the increasing amount of type III collagen was added to reconstituted collagen gel, the diameter of collagen fibrils decreased gradually (**Fig 6**). The difference in diameter between 35% and 40% content, however, was only a few nanometers, i.e., much smaller than the difference between control skin and DNFB-treated skin (35.1 nm *vs* 48.0 nm or 49.6 nm). These results suggest that the decrease in fibril diameter is due to the modifications of type I and/or type III collagen molecules but not to the 5% increase in the content of type III collagen itself.

Collagen of chronically inflamed skin has decreased susceptibility to MMP-2 Susceptibility of collagen to matrix-degrading enzyme was examined by digesting it with MMP-1, MMP-2, and MMP-9. Heat-denatured collagen of DNFB-treated skin showed higher resistance to digestion with MMP-2 under all digestion conditions examined, and a number of partially digested peptide bands were visible after digestion with MMP-2 (**Fig 7**). These bands may have derived from type I or type III collagen. Therefore, these results suggest that denatured type I and/or type III collagen of chronically inflamed skin is more resistant to MMP-2 than control collagen. Native collagen without heat denaturation was not digested with MMP-2 or MMP-9 (data not shown). Susceptibility to MMP-1

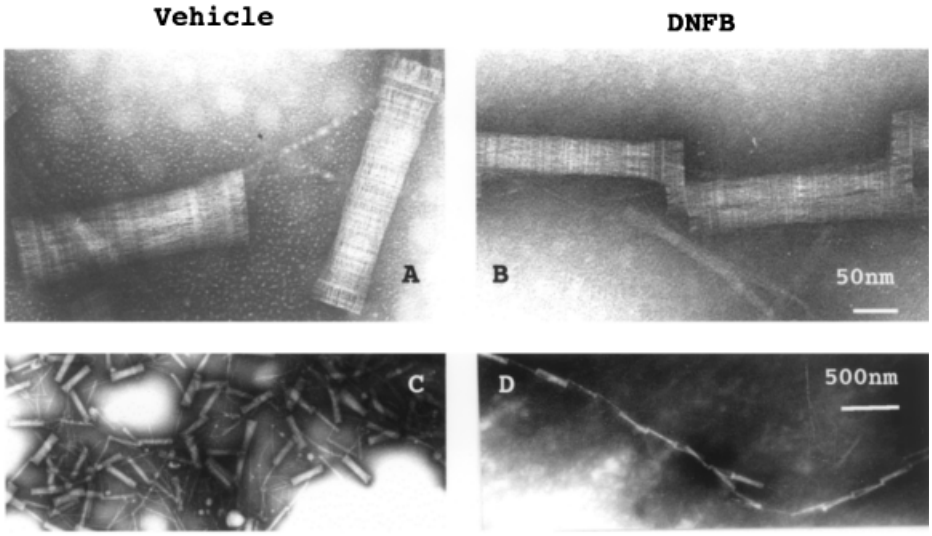

Figure 4. SLS crystallites of collagen of DNFB-treated skin are connected in tandem with a terminal 0.4*D* overlap. SLS crystallites of unpurified collagen from vehicle-treated skin (*A*, *C*) and DNFB-treated skin (*B, D*). *Scale bar.* (*A*), (*B*) 50 nm; (*C*), (*D*) 500 nm.

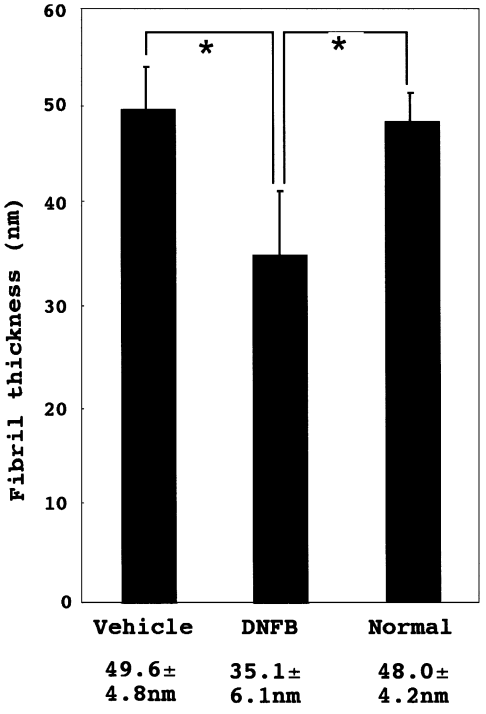

Figure 5. Collagen of DNFB-treated skin forms thin fibrils *in vitro*. Ultrathin sections of collagen fibrils were photographed at 100,000 × , and the diameter was measured by NIH Image (mean ± SD, $n = 600$). $^{*}p < 0.01$.

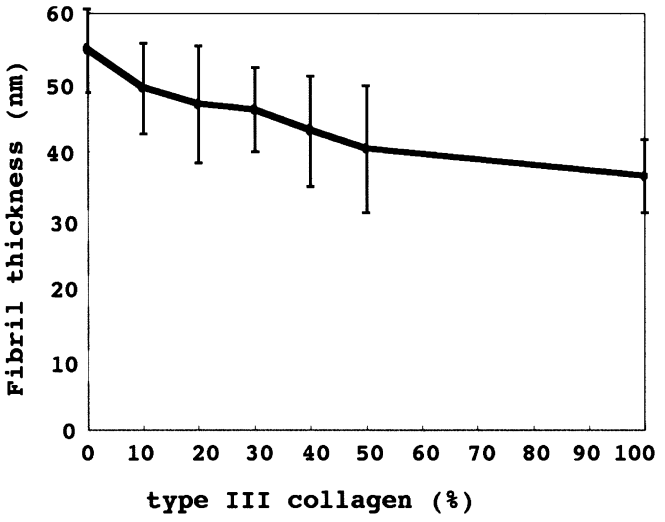

Figure 6. Effect of the content of type III collagen on the diameter of reconstituted collagen fibrils. The diameter of the fibrils was measured as described in the legend for **Fig 5**.

and MMP-9 did not differ among the three groups (data not shown).

Collagen of chronically inflamed skin upregulates secretion of matrix-degrading enzymes We then examined the ability of collagen to regulate the activity of cells by cultivating endothelial cells on the reconstituted collagen gel and measuring the activity of secreted MMP by gelatin zymography. Endothelial cells cultured on a collagen gel secreted latent MMP-2 of 72 kDa and a trace amount of active MMP-2 of 67 kDa, although the latter was not measured precisely. In order to measure the activity of latent MMP-2, culture supernatant was serially diluted and a calibration line was drawn (**Fig 8*A***). The activity of latent MMP-2 was determined in this linear range and expressed relative to the activity of a normal control. There was no significant difference in the activity of latent MMP-2 between normal and vehicle-treated skin (**Fig 9*A***). In contrast, the activity of latent MMP-2 cultured on a gel reconstituted with the collagen of DNFB-treated skin was significantly higher than that of controls (**Fig 9*A***). The number of endothelial cells did not differ among the three groups (data not shown).

When mouse fibroblasts were tested under the same conditions, much active as well as latent MMP-2 was detected. MMP-2 activity was determined using a calibration line of latent MMP-2 (**Fig 8*B***) and active MMP-2 (**Fig 8*C***) and was expressed relative to a vehicle control. Both active and latent MMP-2 were upregulated when fibroblasts were cultured on a gel reconstituted from collagen of chronically inflamed skin (**Fig 9*B***). When normal skin-derived type III collagen was added to a reconstituted gel in a range of 0%–50% content, there was no obvious effect on the activity of secreted MMP-2 (data not shown). No activity of MMP-2 was detected in the presence of ethylenediamine-N,N,N′,N′-tetraacetic acid, disodium salt (data not shown). These results suggest that the upregulation of the secreted MMP-2 may be attributed not to the 5% increase of type III collagen

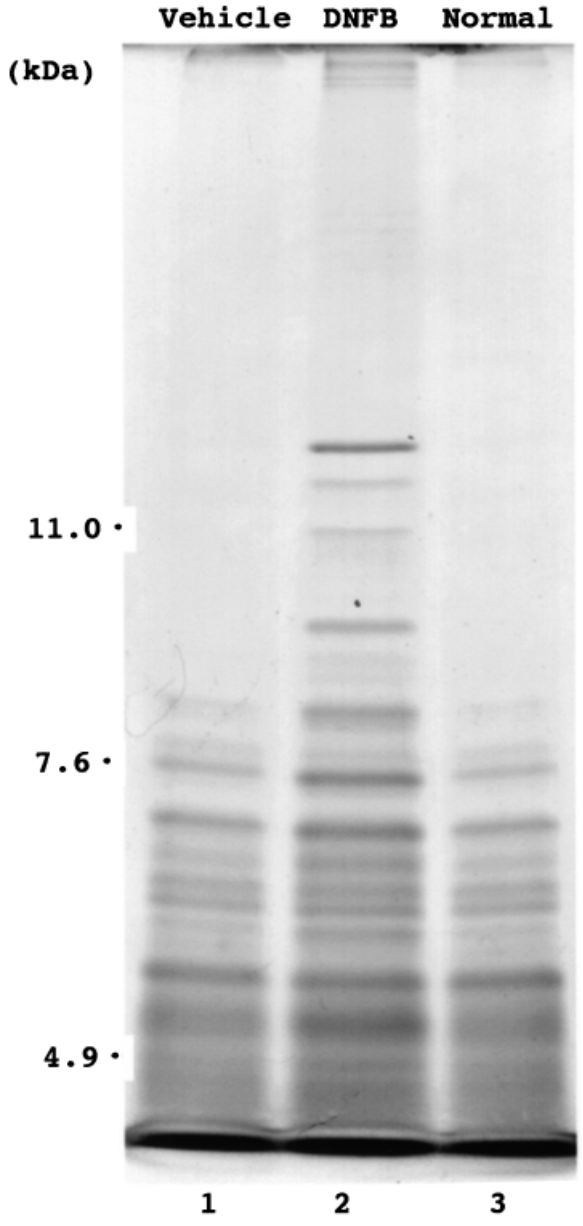

Figure 7. Collagen of DNFB-treated skin has altered susceptibility to MMP-2. Heat-denatured collagen of vehicle-treated skin (*lane 1*), DNFB-treated skin (*lane 2*), and normal skin (*lane 3*) was treated with MMP-2 at 37°C for 24 h and subjected to SDS-PAGE in 7.5% gel under nonreducing conditions.

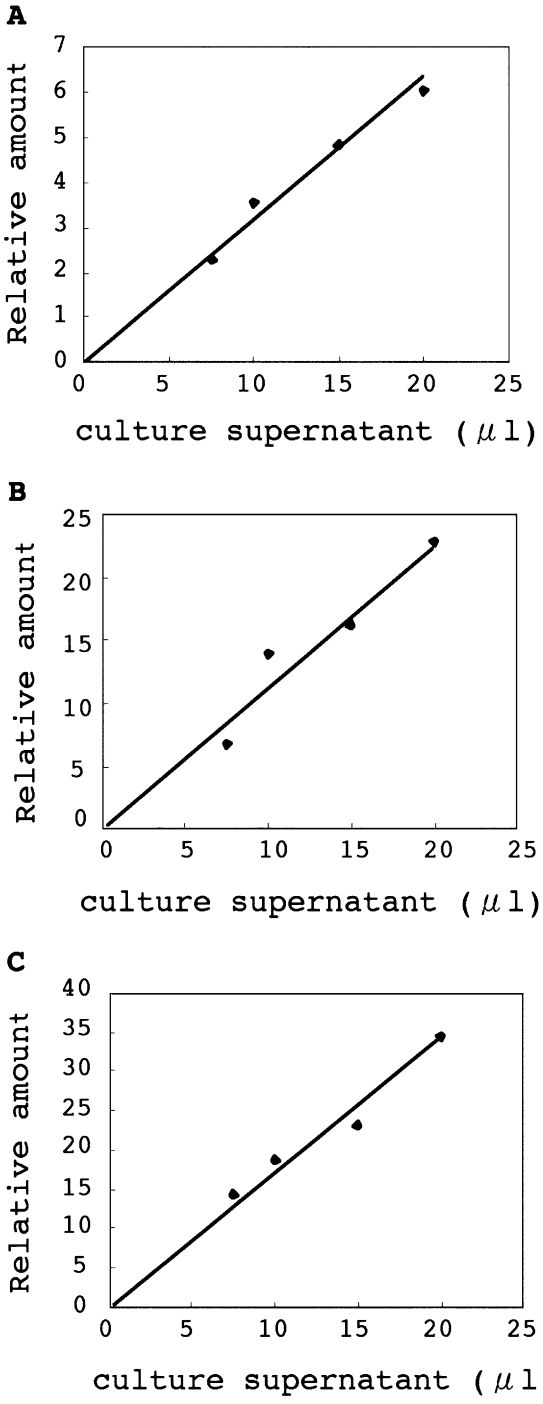

Figure 8. Calibration lines of MMP-2 in culture supernatant of endothelial cells and fibroblasts. Culture supernatant was serially diluted and relative activity of latent MMP-2 from endothelial cells (*A*) and latent (*B*) and active (*C*) MMP-2 from fibroblasts was measured to draw a calibration line.

itself but rather to the modifications of type I and/or type III collagen molecules.

Figure 10 shows the collagen-degrading activity secreted from endothelial cells (**Fig 10*A***) and fibroblasts (**Fig 10*B***). There was no significant difference in the collagen-degrading activity secreted from endothelial cells between the DNFB-treated skin (52.7 ± 3.7) and the vehicle-treated skin (48.8 ± 2.4) and also between the DNFB-treated skin and the normal skin (49.3 ± 2.1) (**Fig 10*A***). In contrast, fibroblasts secreted much larger collagenase activity than endothelial cells, and the secreted collagen-degrading activity was significantly higher for the DNFB-treated skin (96.2 ± 8.6) than the vehicle-treated (76.5 ± 4.5) or the normal skin (78.3 ± 2.5) (**Fig 10*B***). These results suggest that collagen of chronically inflamed skin upregulates the secretion of collagen-degrading enzymes from fibroblasts.

DISCUSSION

In this study, we induced chronic contact dermatitis by repeatedly applying hapten to the ear skin of the mouse, and examined properties of the collagen of chronically inflamed skin. It was found that the collagen of chronically inflamed skin exhibited altered properties such as a higher ratio of hydroxylation of proline and lysine residues, elevated denaturation temperature, intermolecular associations, and decreased thickness of reconstituted fibrils.

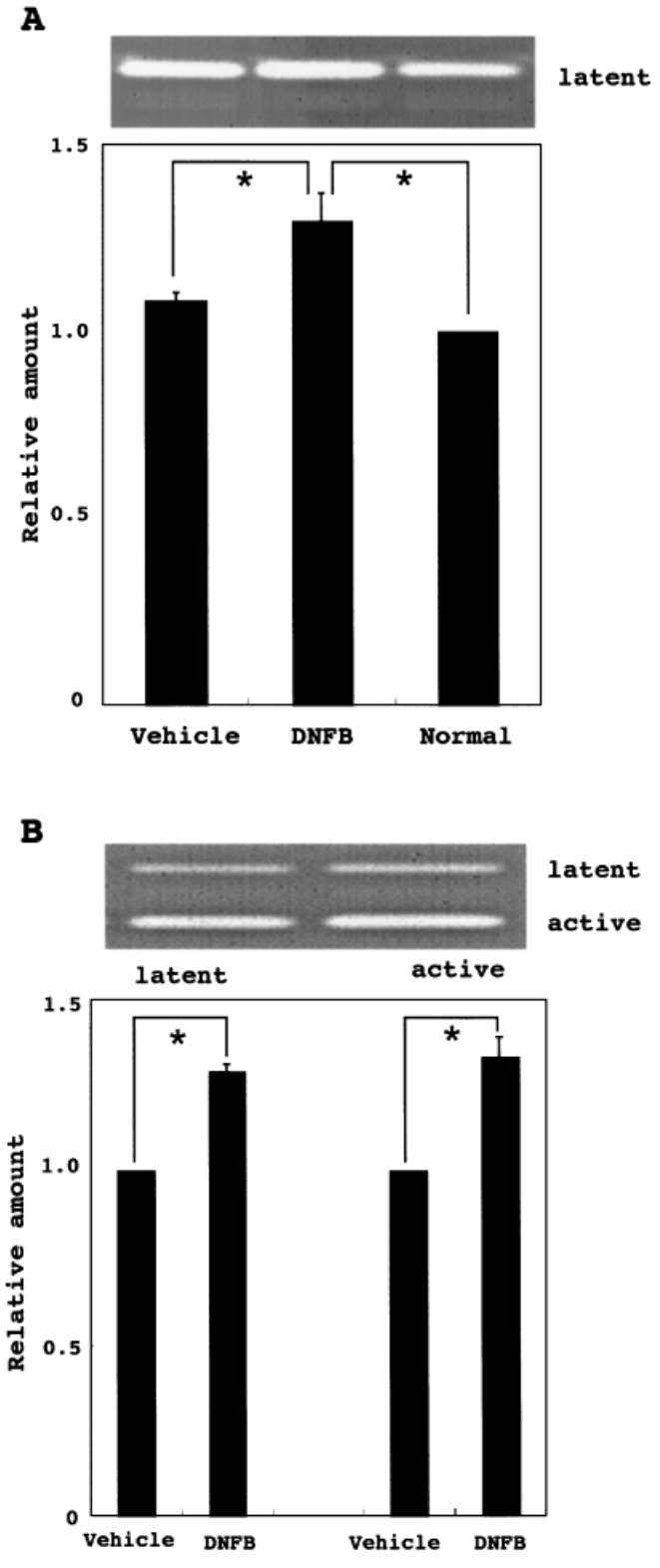

Figure 9. Endothelial cells and fibroblasts secrete an increased amount of MMP-2 when cultured on a gel formed with collagen of DNFB-treated skin. Image of gelatin zymography (*upper figure*) and relative activity of MMP-2 (*lower figure*) in culture medium of endothelial cells (*A*) and fibroblasts (*B*). The relative amount of MMP-2 activity in culture supernatant was determined using the calibration line shown in **Fig 8**. latent, latent MMP-2; active, active MMP-2. Active MMP-2 was not detected by gelatin zymography in the culture medium of endothelial cells. Mean ± SD ($n = 3$). $^{*}p < 0.05$.

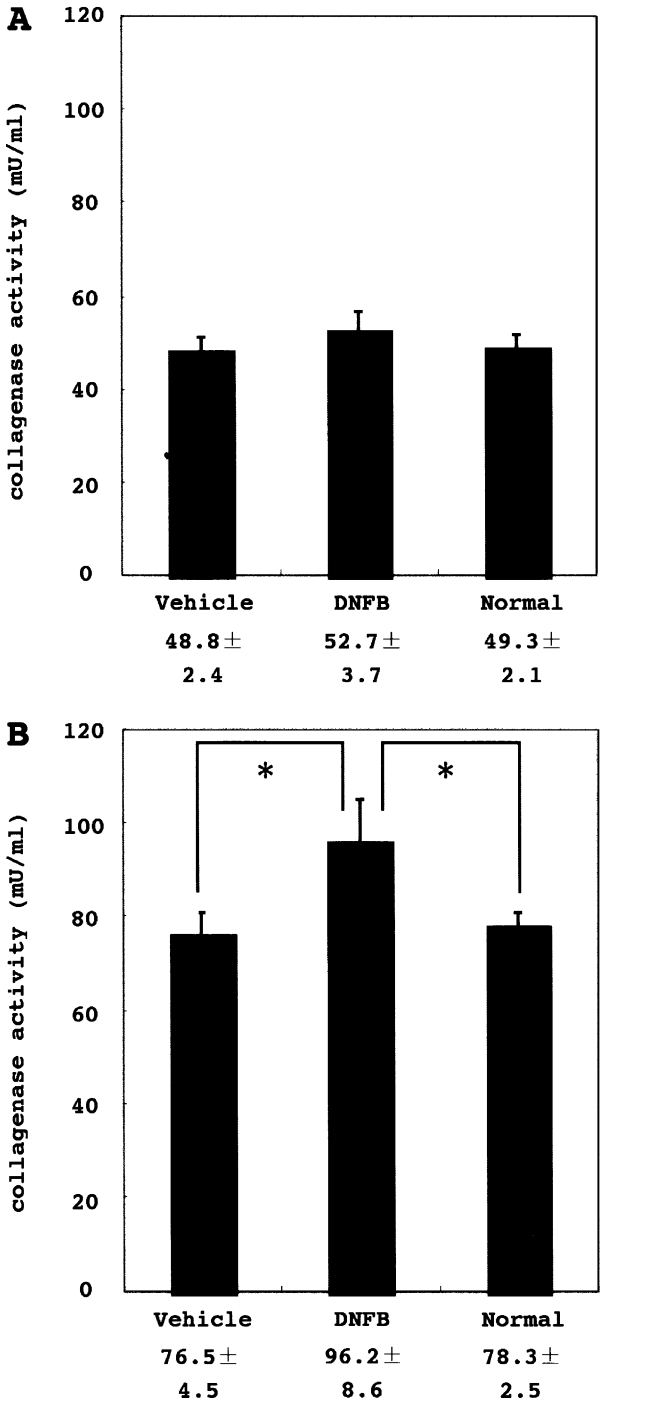

Figure 10. Fibroblasts secrete an increased amount of collagen-degrading activity when cultured on a gel formed with collagen of DNFB-treated skin. Collagen-degrading activity secreted from endothelial cells (*A*) and fibroblasts (*B*). Collagen-degrading activity was measured by determining the amount of fluorescence released from collagen labeled with fluorescein isothiocyanate. Although the mean value of DNFB-treated skin for endothelial cells was slightly higher than for controls, the difference was not significant. Mean ± SD ($n = 5$). $^{*}p < 0.05$.

It was also demonstrated that the collagen had decreased susceptibility to MMP-2 and the ability to upregulate the secretion of matrix-degrading enzymes.

Inflammation and wound healing activate 4-prolylhydroxylase, which hydroxylates proline (Fullana *et al*, 1993). Therefore, it is conceivable that the activity of 4-prolylhydroxylase is increased

by repeated application of DNFB and that the elevated 4-prolyl-hydroxylase activity resulted in a higher ratio of hydroxyproline. Hydroxyproline plays an important role for thermal stability of collagen (Burjanadze, 1979). Therefore, the higher denaturation temperature of collagen in chronically inflamed skin probably results from the increased hydroxylation of proline residues. Over-hydroxylation of lysine residues and/or an increase in glycosylation is reportedly associated with the lower mobility in SDS-PAGE and the decrease of fibril diameter (Butler and Cunningham, 1966; Grant and Jackson, 1968; Spiro, 1969; Torre-Blanco *et al*, 1992; Brinckmann *et al*, 1999). Thus, higher denaturation temperature, lower electrophoretic mobility, and decreased fibril thickness could be explained, at least in part, by an elevated hydroxylation ratio of proline and lysine residues. Although over-modifications of α1(I) and α1(III) were not well elucidated in this study, they are also probably over-modified because their mobility in SDS-PAGE was decreased as for α2(I).

Ultrastructural observations of SLS crystallites revealed that type I collagen of chronically inflamed skin forms unusual 0.4*D*-staggered SLS crystallites. This suggests the possibility that type I collagen is intermolecularly cross-linked in chronically inflamed skin more frequently than in normal skin because similar SLS crystallites were observed when loosely associated collagen was cross-linked with glutaraldehyde and subjected to the formation of SLS crystallites (Kobayashi *et al*, 1985). Although the nature of the cross-link is unknown at present, it may be present in the triple-helical region or in the telopeptide; the cross-link may be located in the terminal 0.4*D* region of the triple helical domain because telopeptide is usually removed when collagen is extracted with pepsin. Alternatively, the cross-link may be present in the telopeptide that might have become resistant to pepsin treatment by the intermolecular cross-link. It is noteworthy that unpurified collagen of DNFB-treated skin formed 0.4*D*-staggered crystallites but purified collagen did not. This suggests that cross-linked collagen was removed by salt fractionation.

The collagen of chronically inflamed skin was more resistant to digestion with MMP-2 than control collagen after denaturation, resulting in the formation of partially digested collagen peptides. The altered susceptibility to MMP-2 may be due to the post-translational over-modifications of collagen molecules found in this study. These results suggest the possibility that partially digested collagen peptide is present in chronically inflamed skin in larger amounts than in normal skin. It has been reported that the collagen-derived peptide shows a biologic activity that is not found in its native state (O'Reilly *et al*, 1997). Therefore, it is tempting to speculate that the partially digested collagen peptide in chronically inflamed skin plays some role in chronic inflammation or its repair. Further studies are required to address this issue.

MMP is responsible for the remodeling of connective tissues during inflammation, morphogenesis, and tumor invasion, and its activity is considered to be the rate-limiting step in extracellular matrix degradation (Docherty and Murphy, 1990; Matrisian, 1990; Woessner, 1991). MMP-2 plays an important role for inflammation (Turck *et al*, 1996) and tumor cell invasion (Itoh *et al*, 1998). Actually, in chronically inflamed skin from which collagen was extracted in this study, MMP-2 was upregulated markedly in comparison with acutely inflamed skin or normal skin (Kusubata *et al*, 1999). This study clearly shows that collagen in chronically inflamed skin has the ability to upregulate the secretion of MMP-2 by endothelial cells and fibroblasts *in vitro*. Thus, the collagen may play a part in upregulating MMP-2 *in vivo* in chronic contact dermatitis. Native type I collagen is degraded by collagenases such as MMP-1, MMP-8, and MMP-13. In this study, it was shown that the collagen of chronically inflamed skin upregulates the secretion of collagen-degrading activity from fibroblasts. Taken together, these results suggest that the collagen in chronically inflamed skin has the ability to upregulate the secretion of matrix-degrading enzymes such as MMP-1, MMP-13, or MMP-2, and thus may accelerate the turnover of collagen in chronically inflamed skin.

It has been reported recently that collagen of aged animals is post-translationally over-modified (Ebihara *et al*, 1999) and that it forms fibrils of thinner diameter compared with collagen of younger animals (Sato *et al*, 2000). The expression of integrin α2β1, a major receptor for collagen in fibroblasts, is lower when cells are cultured on collagen fibrils reconstituted from collagen of aged animals (Sato *et al*, 2000). Thus, it seems possible that fibroblasts cultured on collagen of chronically inflamed skin express a smaller amount of integrin α2β1, and that altered signal transduction through α2β1 results in upregulation of MMP-2.

In connective tissues, chronic inflammation is observed in various diseases including rheumatoid arthritis, periodontal disease, and atopic dermatitis. The chronic wound is distinct from acute wounds with respect to the state of fibronectin in wound fluid (Rao *et al*, 1995). Furthermore, collagenase-3 (MMP-13) is expressed in chronic ulcers by stromal cells but not in normally healing wounds (Vaalamo *et al*, 1997). The expression of fibronectin, tenascin-C, fibulin-1, and fibulin-2 changes during the development of chronic contact dermatitis (Kusubata *et al*, 1999). Thus, chronic disease is probably associated with an altered turnover and architecture of extracellular matrix components. Our study suggests that collagen is over-modified in chronically inflamed skin. This modified collagen has an altered susceptibility to MMP-2 and upregulates the secretion of matrix-degrading enzymes. These altered properties of collagen may affect the pathogenesis of chronic contact dermatitis or its repair. Further studies on the extracellular matrix in chronic diseases may reveal a new aspect of the role of the extracellular matrix in the pathogenesis of chronic diseases.

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