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Matrix Metalloproteinase-1 is the Major Collagenolytic Enzyme Responsible for Collagen Damage in UV-irradiated Human Skin¶

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ABSTRACT

Punch biopsies of human skin were obtained 1 day after irradiation with two minimal-erythema doses (MED) from either a UVB light source or a Solar Simulator and incubated in organ culture for 72 h. Organ culture fluids obtained at 24, 48 and 72 h were analyzed for collagenolytic activity and for reactivity with antibodies to matrix metalloproteinase-1 (MMP-1; interstitial collagenase) and MMP-13 (collagenase-3). High levels of collagenolytic activity were seen in organ culture fluid from skin exposed to either light source. MMP-1 was strongly induced in parallel, increasing from less than 100 ng/ml in organ culture fluid from control skin to approximately 1.1 μg/ml in culture fluid from UV-treated skin. Whereas most of the detectable MMP-1 in control culture fluid was represented by the latent form of the enzyme, approximately 50% of the enzyme was present as the active form in organ culture fluid of UV-exposed skin. In contrast, there was no detectable MMP-13 in control organ culture fluid and very little change after UV exposure (less than 100 ng/ml in both cases). Finally, neutralization studies with a blocking antibody to MMP-1 removed 95 ± 4% of the collagenolytic activity in the organ culture fluid from UV-treated skin. These findings strongly implicate MMP-1 rather than MMP-13 as the major collagenolytic enzyme responsible for collagen damage in photoaging.

INTRODUCTION

The matrix metalloproteinases (MMP) are a family of enzymes that together have the capacity to degrade virtually every component of the extracellular matrix (1,2). Although several MMP are expressed in mammalian skin, only the three collagenolytic enzymes—*i.e.* MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3)—can degrade native, fibrillar Type-I and Type-III collagen.

Studies conducted over the past several years have shown that exposure of the skin to UV radiation results in the upregulation of several different MMP, including those with collagenolytic activity (3,4). The repeated upregulation of these collagen-degrading enzymes over years or decades is thought to underlie the collagen damage that is one of the hallmarks of photoaging. However, the contribution of each of the individual collagenolytic enzymes to the overall connective tissue destruction seen in severely photodamaged skin is not known. Recent studies suggest a limited role for MMP-8 (5). Although this enzyme was induced by UV light, its upregulation was minimal. Furthermore, the time course for MMP-8 expression in UV-irradiated tissue did not correlate with the time course for buildup of collagen fragments in the UV-exposed skin. On the basis of these observations it was concluded that although MMP-8 was present in the skin after UV irradiation, it probably contributed little to the overall structural damage to collagen in photoaging.

The present studies continue our effort to understand the role of individual MMP in photoaging. Here we describe studies in which expression and function of MMP-1 and MMP-13 were directly compared in UV-treated skin. Our studies show that MMP-1 was strongly induced by UV exposure, whereas MMP-13 was either not detectable or detected at a very low level. Concomitant with upregulation of MMP-1 was activation of the latent enzyme. Finally, and most importantly, our studies show that neutralization of MMP-1 removed virtually all the collagen-degrading activity elaborated by UV-treated human skin. Taken together with our previous observations (5), these studies suggest that MMP-1 is the major collagenolytic enzyme responsible for collagen destruction in severely photodamaged skin.

MATERIALS AND METHODS

Preparation of organ culture fluid from irradiated skin. Healthy adults with light skin pigmentation (three female, three male; age range 25–45 years) were irradiated on the hip with light from four F36T12 ERE-VHO tubes as described elsewhere (4). Wavelengths below 290 nm were removed by a Kodacel TA401/407 filter (Eastman Kodak Co., Rochester, NY). An IL1400 phototherapy radiometer and SED240/UVB/UV photodetector (International Light, Newbury, MA) were used to monitor irradiation intensity. Power output distribution in the UV spectrum was 63% UVB (290–320 nm), 25% UVA2 (320–340 nm) and 12% UVA1 (340–400 nm). The UV dose that caused barely perceptible skin reddening (minimal erythema dose [MED]) was determined for each subject as

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Abbreviations: KBM, keratinocyte basal medium; MED, minimal-erythema dose; MMP, matrix metalloproteinase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TIMP, tissue inhibitors of metalloproteinases; TTBS, Tween-TBS.

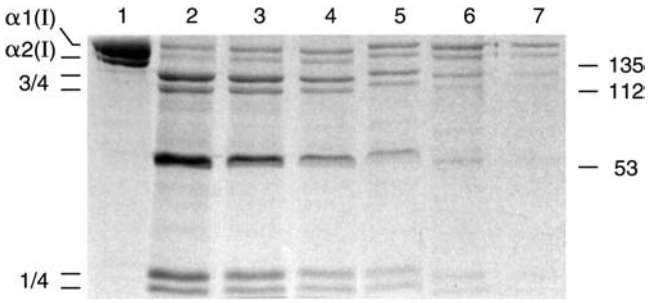

Figure 1. Cleavage of Type-I collagen by organ culture fluid from UV-irradiated human skin. Lane 1 demonstrates the α1 and α2 chains of intact Type-I collagen and the lack of ¾ and ¼ fragments in the collagen stock solution. The stock solution of collagen was separated by SDS-PAGE before polymerization. Lanes 2–7 demonstrate the presence of ¾ and ¼ fragments after exposure of the collagen substrate to decreasing amounts (100–0.5 μL in half-log increments) of a 48 h organ culture fluid from a UVB-exposed skin sample. (Note: The lack of significant amounts of intact collagen in lanes 2–7 reflects the fact that most of the nondigested collagen remains polymerized and does not enter the polyacrylamide gel. Note that the prominent band in the middle of each lane is a protein elaborated in the organ culture fluid).

previously described (4). With this light source, wavelengths in the UVB range of the spectrum are primarily responsible for enzyme induction.

In additional studies, three healthy adults (two female, one male; age range 22–37 years) were exposed to light from a Solar Simulator. To simulate solar radiation, a 450 W xenon arc lamp, filtered with a Schott WG320 filter to remove wavelengths below 290 nm and coupled to a liquid light guide, was used. The spectral output of this source was determined with an OL754 spectroradiometer (Optronic Laboratories, Orlando, FL). The UV spectrum for the xenon arc lamp was 0.00006% UVC, 6.6% UVB, 16.5% UVA2 and 76.8% UVA1. An IL 1440 phototherapy radiometer and a SED240/UVB/UV photodetector (International Light) were used to monitor the light output. As with the UVB light source, the UV dose that caused barely perceptible skin reddening (1 MED) was determined for each subject. With this light source, a combination of wavelengths in the UVB and UVA1 ranges of the spectrum is primarily responsible for enzyme induction.

Duplicate 4 mm punch biopsies of sun-protected (buttock) skin were obtained from the subjects 1 day after exposure to UV irradiation (2 MED) from either light source. The skin biopsies were cut into small pieces (6–8/biopsy) and incubated for 72 h in 1.5 mL of Ca^{2+}-supplemented (1.4 m*M* final concentration) keratinocyte basal medium (KBM) (MA Bioproducts, Walkersville, MD). Incubation was at 37°C under 5% CO_2. Our previous studies have demonstrated that these culture conditions preserve histological structure and biochemical function of human skin for several days (6). At the end of the incubation period, the culture fluid was clarified by low-speed centrifugation and used as the enzyme source. The participation of human subjects in this project was approved by the University of Michigan Institutional Review Board, and all subjects provided written informed consent before their inclusion in the study.

Purified human MMP and other reagents. Human MMP-1 and MMP-13 were obtained from Calbiochem (San Diego, CA). MMP-1 was purified from human rheumatoid synovial fibroblasts as the proenzyme form, and MMP-13 was obtained as a recombinant protein encompassing the proenzyme 60 kDa form. Rabbit polyclonal IgG antibodies to MMP-1 (AB806) and MMP-13 (AB8114) were obtained from Chemicon International (Temecula, CA). These antibodies were used in Western blotting studies. A rabbit polyclonal IgG (R & D Systems, Minneapolis, MN) was used as a control. In addition to these antibodies, a mouse monoclonal anti–MMP-1 antibody with neutralizing activity (Ab-5; IM66) was obtained from Oncogene Research Products (San Diego, CA).

Western blotting. Supernatant fluids from control or UV-exposed skin were analyzed by Western blotting with rabbit polyclonal anti–MMP-1 or anti–MMP-13 antibodies. Purified MMP-1 and MMP-13 served as standards. Samples were separated in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing and reducing conditions and transferred to nitrocellulose membranes. After blocking with a 5% nonfat milk solution in Tris–buffered saline (TBS) at 4°C overnight, membranes were incubated for 1 h at room temperature with either of the rabbit polyclonal antibodies, diluted 1:6000 in 0.5% nonfat milk–0.1% Tween-TBS (TTBS). Thereafter the membranes were washed with TTBS and the bound antibodies detected using the Phototope-HRP Western blot detection kit (New England Biolabs, Beverly, MA). The Western blotting procedure has been described previously (7). Preliminary studies revealed no detectable cross-reactivity between purified MMP-1 and anti–MMP-13 antibodies or between purified MMP-13 and anti–MMP-1 antibodies.

Substrate-embedded enzymography. SDS-PAGE substrate-embedded enzymography (zymography) was used to identify enzymes with collagenase and gelatinase activities. Assays were carried out exactly as described in a previous report (8). In brief, denatured but nonreduced culture fluid samples were resolved in 7.5% SDS-PAGE gels prepared with the incorporation of gelatin (1 mg/mL) or β-casein (1 mg/mL) before casting. After electrophoresis, gels were washed twice for 15 min in 50 m*M* Tris buffer containing 1 m*M* Ca^{2+}, 0.5 m*M* Zn^{2+} and 2.5% Triton X-100. The gels were then incubated overnight in Tris buffer with 1% Triton X-100 and stained the following day with Coomassie Brilliant Blue 250-R. After destaining, zones of enzyme activity were detected as regions of negative staining against the dark background. Volumes of 5–35 μL of undiluted culture fluid were normally used for these assays; zones of activity were proportional to the quantity of culture fluid used. Gelatin zymography is useful for detection of MMP-2 (72 kDa gelatinase A) and MMP-9 (92 kDa gelatinase B). MMP-13 can also be detected by gelatin zymography as a zone of hydrolysis beneath the active MMP-2 band. β-Casein zymography is useful for detection of MMP-1. The latent form of the enzyme appears as a doublet in the 54 kDa region of the gel, and if active forms are present, they show up as a doublet in the 45 kDa region.

Degradation of native collagen. Rat tail collagen (4.7 mg/mL in 1 *N* HCl) (BD Biosciences, Bedford, MA) was diluted to 1 mg/mL in culture medium consisting of serum-free, Ca^{2+}-supplemented KBM. The collagen solution was made isotonic by addition of an appropriate amount of 10× concentrated Hank balanced salt solution and the pH brought to 7.2. The collagen was dispensed into glass tubes (0.5 mL/tube) for use in collagen digestion assays. Degradation of the native collagen was achieved by exposing it to the human skin organ culture fluid for 18 h at 37°C. Intact collagen exposed to buffer alone served as control. At the end of the incubation period, the supernatant fluids from the control or treated collagen were assayed. Collagen fragments released into the supernatant fluid were resolved by SDS-PAGE (8.5% gel) and staining with Coomassie Brilliant Blue 250-R. This procedure has been described previously (9,10).

Neutralization of MMP-1. Collagen was prepared as described above. Organ culture fluid from UV-treated skin was used without any additional treatment. Purified MMP-1 was activated by exposure to 1 μg/mL trypsin for 5 min followed by inactivation of the trypsin with 10 μg soybean trypsin inhibitor. Organ culture fluid (100 μL) and activated MMP-1 (100 ng) were each dispensed into duplicate tubes. One tube of each pair was exposed to the neutralizing antibody (10 μg), whereas the second tube of the pair received buffer alone. After incubation for 15 min at 37°C, 0.5 mL of the freshly prepared collagen solution was added to each tube and incubated for 18 h at 37°C. Intact collagen not exposed to either the organ culture fluid or the purified enzyme was also incubated for 18 h as a control. At the end of the incubation period, the supernatant fluids from the control or treated collagen samples were removed. Collagen fragments released into the supernatant fluid were assessed as above. Stained images were digitized and scanned. The digitized ¾ and ¼ fragments were quantified and used as a measure of collagenolytic activity. Reduction of fragment production is therefore a measure of enzyme neutralization.

RESULTS

Collagenolytic activity in culture fluid from UV-treated skin

In the first series of experiments, organ culture fluids prepared from skin samples irradiated with the UVB light source were assessed for ability to degrade intact fibrillar collagen. Shown in Fig. 1 are results obtained with a 48 h culture fluid from one individual. In the absence of culture fluid (lane 1), intact α1 and α2 chains of Type-I collagen were seen. No detectable fragments were present. Lanes 2–7 demonstrate the capacity of culture fluid from the UV-exposed skin to degrade the intact collagen chains to produce

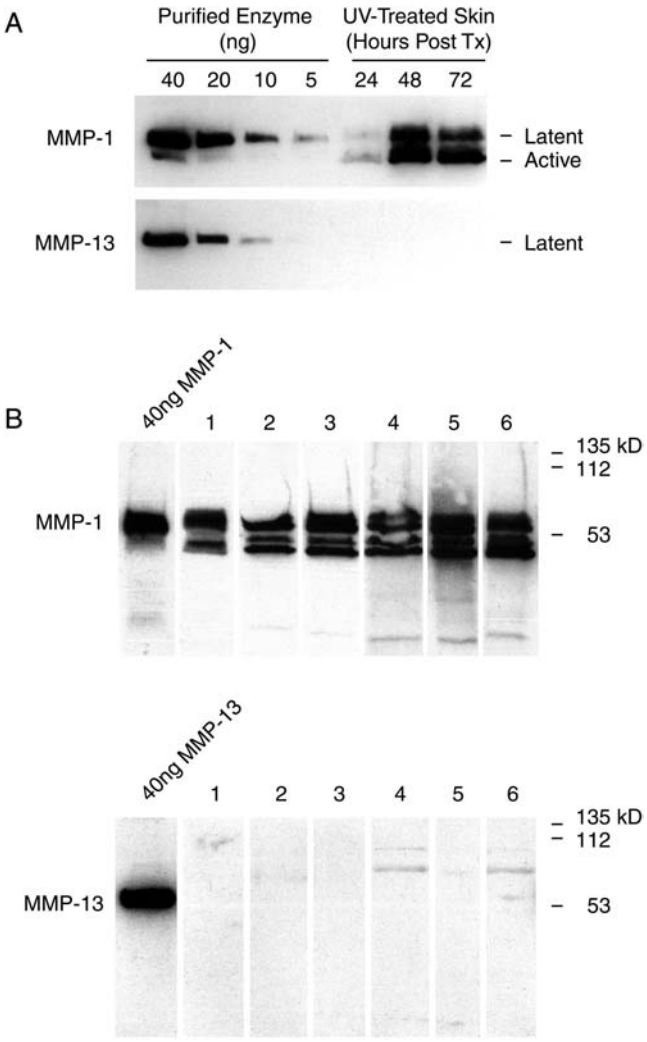

Figure 2. Western blot immunoreactivity of organ culture fluid with antibodies to MMP-1 and MMP-13. A: Varying amounts of purified MMP-1 and MMP-13 are presented on the left. Each of the lanes on the right contains 35 μL of organ culture fluid obtained after 24, 48 or 72 h of incubation. The results shown in this panel were obtained with organ-cultured skin from a single volunteer. B: Forty nanograms of purified MMP-1 and MMP-13 are presented on the left. Lanes 1–6 contain 35 μL of organ culture fluid from six individuals obtained after 48 h of incubation. (Note: detectable MMP-13 immunoreactivity is present in lanes 4 and 6, but the molecular weight of the major product [approximately 70 kDa] is greater than that of the recombinant standard.

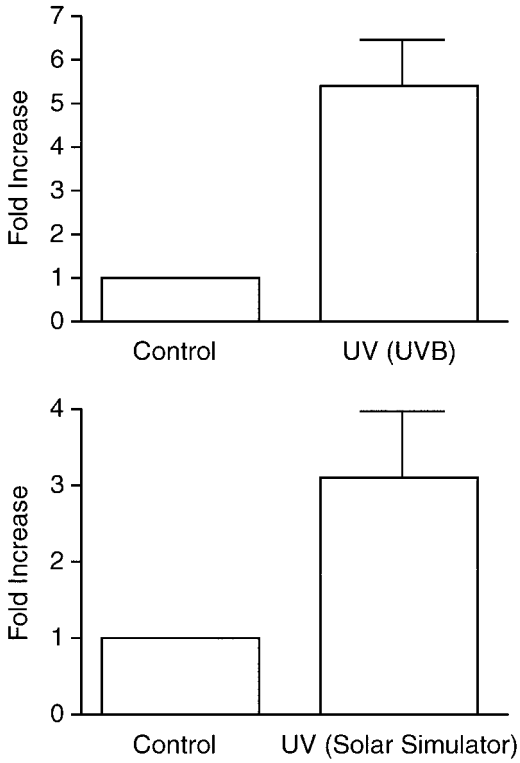

Figure 3. Up-regulation of MMP-1 in skin exposed to the UVB light source or to light from the Solar Simulator. Immunoreactive bands from 48 h control and irradiated skin samples were digitized. Values obtained with control samples from each individual were arbitrarily set at 1.0 and values obtained after irradiation compared with this. Upper panel: UVB light source. Lower panel: Solar Simulator. Differences between the control and irradiated samples were compared using Student's *t*-test. Values shown for the UVB source were statistically different at the 0.01 level. Values shown for the Solar Simulator were statistically different at the 0.07 level.

visible ¾ and ¼ fragments. Collagen fragmentation was dose-responsive over the range of 0.5 to 100 μL of culture fluid. The results shown here with skin from one volunteer were representative of results from all the individuals examined.

Immunoreactive MMP-1 and MMP-13 in culture fluid from UV-treated skin

The same culture fluids were examined by Western blotting for reactivity with antibodies to MMP-1 and MMP-13. The results are shown in Fig. 2. Panel A shows a representative time course for induction of MMP-1 and MMP-13 after UV irradiation. Anti–MMP-1 reactivity was present in the culture fluid after 24 h of incubation but was much stronger after 48 and 72 h. Both latent and active forms of the enzyme were detected. The presence of active enzyme forms is not surprising, given the level of collagen-degrading activity noted in the same culture fluids (see Fig. 1). Panel B of Fig. 2 compares Western blot results from six subjects (48 h postirradiation). Strong anti–MMP-1 reactivity was observed in the organ culture fluids from all six subjects. Both active and latent enzyme forms were present in all the culture fluids. Comparison of organ culture fluid from UV-irradiated human skin with purified fibroblast-derived MMP-1 (run in varying amounts in Western blots) indicated that in the 48 h culture fluids (the highest enzyme levels were achieved at 48 h) the average amount of MMP-1 in the culture fluids was 1.1 ± 0.5 μg/mL, with the maximal level being approximately 1.8 μg/mL.

In a second series of studies, three additional volunteers were irradiated with 2 MED of light from a Solar Simulator. Their culture fluids (48 h) were examined by Western blotting for reactivity with antibodies to MMP-1 and MMP-13. As was seen with the UVB light source, Solar Simulator light induced significant upregulation of MMP-1 in these samples but did not result in induction of MMP-13 (not shown).

MMP-1 immunoreactive bands in the 48 h culture fluids from all nine patients (six from UVB experiment and three from the Solar Simulator experiment) were digitized. Comparing the number of pixels in bands from UV-treated *versus* control organ culture fluid provided a way to quantify MMP-1 upregulation. The results,

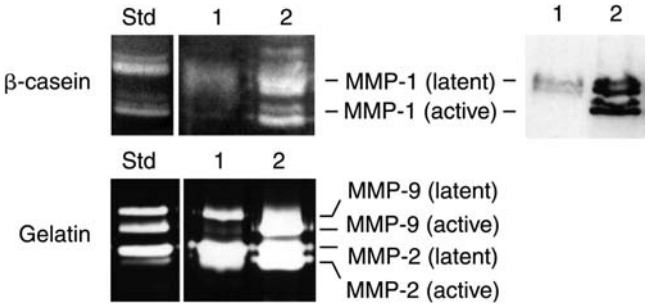

Figure 4. β-Casein and gelatin zymography of organ culture fluid from nonirradiated and UVB-treated human skin. An MMP-1 standard is shown at left in the β-casein zymogram. Lane 1: organ culture fluid from normal (non-irradiated) skin. Lane 2: organ culture fluid from UV-irradiated skin (48 h in culture). A faint, hazy band corresponding to latent MMP-1 can be seen in lane 1 (normal skin) of the β-casein zymogram. Bands corresponding to latent and active forms of MMP-1 can be seen in lane 2 (UV-treated) of the β-casein zymogram. Latent and active bands in β-casein zymography correspond to anti–MMP-1 immunoreactive bands in Western blots (right-side panel). A combination MMP-2 and MMP-9 standard is shown at left in gelatin zymography. Bands corresponding to MMP-9 and MMP-2 can be seen in lanes 1 and 2 of the gelatin zymogram. Bands corresponding to both latent and active MMP-2 are present in both lanes, and the levels are similar. A band corresponding to latent MMP-9 is seen in lane 1, and lane 2 demonstrates bands corresponding to both latent and active MMP-9. MMP-9 reactivity is increased in organ culture fluid from UV-treated skin as compared with control. No band is observed immediately below the active MMP-2 band in either lane. The results shown in this figure were obtained with organ-cultured skin from a single volunteer. The results were consistent among all volunteers.

shown in Fig. 3, indicate that MMP-1 was induced approximately five-fold in response to UVB stimulation and 3.1-fold in response to irradiation with the Solar Simulator. The upregulation by both UV sources was statistically significant, but the difference between the two light sources was not.

Assessment of organ culture fluid from UV-treated skin by β-casein zymography and gelatin zymography

In addition to Western blotting, we also used β-casein zymography and gelatin zymography to characterize enzyme forms present in organ culture fluids from UVB-exposed skin samples. Figure 4 (lane 2) shows results with organ culture fluid from UV-treated skin of one individual. It can be seen from the upper panels that zones of β-casein hydrolysis corresponding to latent and active forms of MMP-1 were present. Lane 1 of the same panel presents results obtained with organ culture fluid from control human skin. Latent MMP-1 was barely detectable, and there was no evidence of active enzyme forms. It can be seen from the figure that enzyme forms detectable in the β-casein zymogram comigrated with forms in the MMP-1 Western blot. The lower panel of Fig. 4 presents results from gelatin zymography. Consistent with past results (3,4), organ culture fluid from UV-treated skin demonstrated significantly increased amounts of latent and active MMP-9 (gelatinase B) as compared with organ culture fluid from control skin. Also consistent with past results (3), there was little difference between UV-treated and control skin in the expression of MMP-2 (gelatinase A). MMP-13, which characteristically appears as a zone of hydrolysis below the active form of MMP-2 (11), was not detected in either the UV-treated or control skin organ culture fluid.

Inhibition of collagen-degrading activity in organ culture fluid of UV-treated human skin

Taken together, the above findings strongly suggest that MMP-1 rather than MMP-13 is responsible for most of the collagen-degrading activity in organ culture fluid of UV-irradiated human skin. To confirm this, organ culture fluid from three UV-treated skin samples (100 μL/sample) was exposed for 15 min to 10 μg of a neutralizing antibody to MMP-1. One hundred nanograms of purified and activated MMP-1 was exposed to the same antibody as a control. At the end of the 15 min incubation period, 0.5 mg of intact Type-I collagen was added to the antibody-treated samples and to appropriate controls. The samples were incubated for 18 h, after which fragmentation of the intact collagen chains to ¾ and ¼ fragments was assessed. Results from studies with organ culture fluid indicated that 95 ± 4% of the activity was removed by antibody treatment (not shown). In parallel studies with purified and activated MMP-1, 88 ± 6% inhibition was achieved ($n = 3$).

DISCUSSION

Extensive damage to the collagenous matrix of the skin is one of the hallmarks of photoaging. Upregulation of MMP over years or decades by chronic or episodic exposure to UV irradiation from the sun is thought to be the major contributor to the collagen damage seen in photoaging (4), but the precise role of individual MMP is not yet known. There are three collagenolytic MMP found in human skin—MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3). Recent studies by Fisher *et al*. (5) provided strong suggestive evidence that MMP-8 is unlikely to play a major role in UV-mediated collagen damage in the skin.

The present study focuses on the other two collagenases (MMP-1 and MMP-13). Our results strongly suggest that of the two enzymes, MMP-1 is likely to play the predominant role. MMP-1 was strongly upregulated in skin after UV exposure, whereas MMP-13 either was not detected or was barely detected. By comparison of organ culture fluid from UV-irradiated human skin with purified fibroblast-derived MMP-1 in Western blots, we estimate that in the 48 h culture fluids (the highest enzyme levels were achieved at 48 h) MMP-1 was present at levels up to 1.8 μg/mL. Interestingly, immunoreactive bands detected in Western blots (as well as zymographic bands in the β-casein zymograms) indicated that a significant proportion of the total enzyme was present as the lower molecular weight active forms rather than as latent forms. What accounts for MMP-1 activation in UV-exposed skin is not known with certainty. Enzyme activation may reflect free radical generation because oxygen free radicals are known to be generated in human skin by UV light (12) and oxidants are known to be potent activators of MMP (13). Alternatively, other MMP as well as serine proteinases such as plasmin have the capacity to activate MMP (14,15). It would be of interest to know if one or more of these enzymes were upregulated by UV exposure. In any event, the presence of active MMP-1 was detected in the Day-1 culture fluid, where total MMP-1 levels were low, as well as at the later time-points. Thus, it is likely that enzyme activation is not simply a consequence of long-term incubation in organ culture. It should be noted in this regard that past studies have demonstrated the presence of active collagenolytic and gelatinolytic activities in UV-irradiated skin (3,4). In contrast, MMP-1

detected in organ culture fluid from normal human skin was present in much smaller amounts and was always present as the latent form only.

Exposure of Type-I collagen to organ culture fluid from UV-treated skin resulted primarily in generation of the ¾ and ¼ fragments. This is of interest because in addition to the upregulation of MMP-1, UV irradiation also resulted in the induction and activation of MMP-9 (3,4) (see Fig. 4). Because MMP-9 effectively removes collagen fragments generated by exposure of intact collagen to MMP-1 (10), the question arises as to why the enzyme (MMP-9) is apparently not effective in whole skin organ culture fluid. Two possibilities come to mind. First, it may be a question of stoichiometry. Each α1 or α2 chain of Type-I collagen provides a single molecule of substrate for MMP-1. In contrast, each ¾ and ¼ fragment produced by MMP-1 presents multiple sites for cleavage by MMP-9. Thus, the effective rate of bond cleavage would need to be much higher with MMP-9 than MMP-1 for the gelatinolytic enzyme to keep up with the collagenase. Alternatively, the level of MMP-9 activity may be lower in the organ culture fluid than what it appears to be from gelatin zymography because of inhibitors present in the culture fluid. Although tissue inhibitors of metalloproteinases (TIMP) are not completely selective among MMP, there is a degree of specificity. In particular, TIMP-1 has a high affinity for MMP-9 (16–18). We have already demonstrated that virtually all the MMP inhibitor activity detected in human skin organ culture fluid is caused by TIMP-1 (7,19). Thus, it may be that the available MMP inhibitor is bound up with MMP-9 while leaving most of the MMP-1 free. Additional studies will be needed to distinguish between these two possibilities, which are, in any case, not mutually exclusive.

In recent studies, we demonstrated that when human skin fibroblasts were incubated on an *in vitro* matrix consisting of MMP-1–damaged Type-I collagen, production of Type-I procollagen was reduced by as much as 80% as compared with procollagen production on an intact collagen matrix (10). Under conditions in which procollagen production was inhibited, it was estimated that fragments were introduced in 30–40% of the collagen molecules. The major degradation products were the ¾ and ¼ fragments. When exogenous MMP-9 was included in the incubation buffer along with MMP-1, the major collagenous fragments were further degraded and cleared. Under these conditions, loss of procollagen synthesis was not observed. On the basis of these recent results, we hypothesized that high molecular weight fragments of collagen served to inhibit new collagen synthesis. The present studies, taken in conjunction with these previous results, suggest that rather than being an *in vitro* observation only, the production of high molecular weight fragments of Type-I collagen by MMP-1 under conditions in which they cannot be effectively cleared by MMP-9 occurs *in vivo*. The lack of procollagen production, which is one of the major hallmarks of severely photodamaged skin (20,21) may therefore reflect a response to the presence of fragmented collagen in the severely damaged skin.

In summary, MMP-mediated collagen damage is a characteristic feature of photodamaged human skin. Of the three collagenolytic enzymes present in the skin, MMP-1 (interstitial collagenase) appears to be the predominant enzyme responsible for degradation of the collagen.

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