Experimental Dermatology 2003: 12 (Suppl. 2): 35–42
Blackwell Munksgaard . Printed in Denmark

EXPERIMENTAL DERMATOLOGY
ISSN 1479-1250

Tretinoin reverses upregulation of matrix metalloproteinase-13 in human keloid-derived fibroblasts

Uchida G, Yoshimura K, Kitano Y, Okazaki M, Harii K. Tretinoin reverses upregulation of matrix metalloproteinase-13 in human keloid-derived fibroblasts.
Exp Dermatol 2003: 12 (Suppl. 2) : 35–42. © Blackwell Munksgaard, 2003

Abstract: Keloids are skin abnormalities that are characterized by excessive deposition of collagen bundles in the dermis. Patients with keloids complain not only about their cosmetic appearance, but also about continuous itching and/or tenderness associated with chronic inflammation. Degradation of extracellular matrix (ECM) may be upregulated, associated with the expansion of keloids into circumferential skin, and high metabolic activity of keloid tissues may be due to increased matrix metalloproteinase (MMP) activity. Based on these hypotheses, we examined differences in expression of MMP-1, MMP-8, and MMP-13 between keloid-derived and normal dermal fibroblasts. Since retinoids are potent inhibitors of MMPs in the treatment of photoaged skin and cancers, we also examined whether or not tretinoin affects MMP expression of keloid-derived fibroblasts. The results of real-time polymerase chain reaction and ELISA demonstrated significant upregulation of MMP-13 and significant downregulation of MMP-1 and MMP-8 in keloid-derived fibroblasts, at both mRNA and protein levels. MMP-1 mRNA expression in the control group was significantly upregulated after the addition of tretinoin, whereas no significant change was observed in the keloid group. MMP-8 mRNA expression in the control group was significantly upregulated by tretinoin, with the peak at 12 h, while no significant change was observed in the keloid-derived fibroblasts. In contrast, the remarkably elevated MMP-13 mRNA expression in the keloid group was significantly suppressed, with the peak suppression at 12 h after addition of tretinoin, while MMP-13 mRNA expression in the control group was not significantly changed. The decrease in MMP-1 and MMP-8 may contribute to accumulation of type I and type III collagen in keloid tissues, and this mechanism may be modulated by molecular interaction with MMP-13. Tretinoin appeared to reverse the abnormal expression profile of MMPs in keloid-derived fibroblasts, such as markedly elevated expression of MMP-13, partly through inactivation of AP-1 pathway. The present results suggest that tretinoin may be clinically useful to improve the chronic inflammation seen in keloids and prevent expansion of keloid tissues into circumferential normal skin.

Gentaro Uchida, Kotaro Yoshimura, Yukie Kitano, Mutsumi Okazaki and Kiyonori Harii

Department of Plastic and Reconstructive Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Keywords: chemoprevention – fibroblast – keloid – matrix metalloproteinases (MMPs) – tretinoin (all-*trans* retinoic acid)

Kotaro Yoshimura MD
Department of Plastic and Reconstructive Surgery
Graduate School of Medicine
University of Tokyo
7-3-1 Hongo
Bunkyo-ku
Tokyo 113-8655
Japan.
Tel: +81 3 5800 8670
Fax: +81 3 5800 6929
E-mail: yoshimura-pla@h.u-tokyo.ac.jp

Introduction

Keloids are skin abnormalities characterized by excessive deposition of collagen bundles in the dermis. In keloids, the normal wound healing process is derailed from the normal, resulting in impairment of the balance between production and degradation of extracellular matrix (ECM) components such as collagens (1). Since fibroblasts play a leading part in production of ECMs, it is thought that there is a difference in the cellular function between keloid-derived fibroblasts and normal ones. However, accumulating data have shown that there is no significant difference in culture growth, cell size, population density, and karyotype between these fibroblasts (2).

During normal wound repair, type III collagen appears at day 2–3, followed by type I collagen at day 6–7 (3). The total amount of type I and III collagen increases over time, whereas the proportion of type III collagen decreases from 60% at 1 week after wounding, to 28% in the mature scar (4). In keloids, however, the relative amount of type III collagen remains high compared with normal scars or normal skin (5). The ratio of α-1(I)-procollagen (a precursor of type I collagen) mRNA to α-1(III)-procollagen (a precursor of type III collagen) mRNA is markedly elevated in keloid-derived fibroblasts compared with that in normal-tissue-derived fibroblasts *in vitro* (6). The same tendency is also observed in keloid tissues *in vivo* (7). There seems to be a discrepancy between excessive accumulation of type III collagen in keloid tissues and an elevated mRNA level of type I procollagen in biosynthesis, which remains unknown. This discrepancy is explicable if cytologic aberrations occur at the level of collagen fiber degradation, especially type III collagen. However, previous studies have only shown a normal (8), decreased (9), or increased (10) collagenase activity (more accurately, degradation activity of type I collagen); no studies have demonstrated altered expression of each type of collagenase in keloid tissues or keloid-derived fibroblasts.

Currently, collagenases are categorized as matrix metalloproteinase (MMPs), which are a group of endoproteinases with a divalent Zn^{2+} at the active site involved in ECM remodeling. MMP-1 (also known as interstitial collagenase or collagenase 1), MMP-8 (neutrophil collagenase, collagenase 2), and MMP-13 (collagenase 3) are the only mammalian enzymes recognized for their unique ability to cleave the triple helical domain of fibrillar collagen types I, II, and III (11). However, each collagenase differs in the extent to which it cleaves these fibrillar collagen subtypes *in vitro*. MMP-1 preferentially degrades type III collagen, whereas MMP-8 has its greatest activity on type I collagen (12). Neither MMP-1 nor MMP-8 appears to have any significant activity against type II and IV collagen. MMP-13 is the most recently discovered human collagenase, which can degrade all fibrillar collagen subtypes with almost equal efficacy, and is the only collagenase with significant activity against type II and IV collagen (13).

Before establishing the novel classification of collagenases described above, fibroplastic lesions due to deposition of ECM components such as collagen fibers had been classified into two groups; increased-level and decreased-level, according to their collagenase activities. Rheumatoid arthritis, osteoarthritis, periodontal diseases, otitis media cholesteatoma, and malignant tumors belong to the former, while pulmonary fibrosis, hepatic fibrosis, hepatic cirrhosis, and systemic sclerosis belong to the latter (11). We thought that, to decide the direction of treatment for keloids, it was essential to determine whether keloids belong to the former group or the latter one. The activity of MMPs is regulated at three levels; transcription, zymogen activation, and inhibition of proteolytic activity (11). Regarding regulation at the transcriptional level, most MMPs are induced through activation of nuclear AP-1 transcription factor (14–16). The AP-1-dependent activation of inducible MMPs is potently inhibited by glucocorticoids (17) and retinoids (18) at the transcriptional level. For regulation at the extracellular zymogen activation level, latent precursors or zymogens of most MMPs are proteolytically activated via exposure of the catalytic site (19). As for the regulation at the level of inhibition of proteolytic activity, nonspecific inhibitors such as α_2-macroglobulin and α_1-antiprotease, and specific inhibitors such as tissue inhibitors of metalloproteinases (TIMPs), are responsible for the inhibition (20).

It has been reported that MMP-1 and MMP-8 activities are upregulated in skin photoaged by repeated exposure to ultraviolet irradiation (21,22). However, tretinoin suppressed upregulated MMP-1 in photoaged skin at the transcriptional level, probably via anti-AP-1 effects (23).

The activity of MMPs is also intimately correlated with the invasive or metastatic ability of malignant tumor cells (24,25). For skin malignancies especially, degradation of ECM is the first step to local invasion and metastasis. Thus, basic and clinical studies have been performed with the aim of chemoprevention of ECM degradation in malignant melanoma, basal cell carcinoma, and squamous cell carcinoma (26–28), and chemoprevention of cell growth. Retinoids are the subject of increasing interest as an effective means of controlling upregulated MMP activity of malignant tumor cells and inhibiting the advancement of tumors (27). It has been reported that retinoids suppress MMP-1 and MMP-8 activity in these malignant tumor cells *in vitro* (28).

Thus, we hypothesized that degradation of ECM may be upregulated during the expansion of keloids into circumferential skin, and that the high metabolic activity of keloid tissues (29) may be due to increased MMP activity, which may contribute to the continuous itching and/or tenderness associated with chronic inflammation seen in keloids (30). Based on these hypotheses, we examined differences in expression of MMP-1, MMP-8, and MMP-13 between keloid-derived and normal dermal fibroblasts. Since retinoids

are potent inhibitors of MMPs in the treatment of photoaged skin and cancers, as described above, we also examined whether or not tretinoin affects MMP expression of keloid-derived fibroblasts.

Materials and methods

Clinical specimens

Twelve specimens of keloid (keloid group), diagnosed on the basis of their clinical appearance, anatomic location, etc., were excised at the Department of Plastic and Reconstructive Surgery, University of Tokyo Hospital. As a control group, 12 normal skin samples, matched to the site of predilection for keloids (scapular area, shoulder, and upper arm), were also excised during the plastic surgery.

Part of each tissue sample was used to establish a primary cell culture, and the rest was used for histopathologic diagnosis. All keloid samples displayed the diagnostic histopathologic characteristics of keloids. No hypertrophic scars were included in the materials. The clinical data of the keloid group and the control group are shown in Table 1. No significant difference in age between the two groups was observed (unpaired Student's *t*-test; $P = 0.4907$). All the biopsies were taken in accordance with the Declaration of Helsinki.

Primary dermal fibroblast cultures

The primary dermal fibroblast cultures from the keloids ($n = 12$) and control skin samples ($n = 12$) were established using explants (31). For primary culture of keloid fibroblasts, marginal portions of keloid lesions were used. Briefly, after removal of the reticular layer of the dermis and epidermis from total skin samples, the surface side of the papillary layer was attached to the culture dish, then the culture medium was added and a cell culture was started (37°C, 5% CO_2). Subculture was performed 2 weeks after primary culture, when cell culture reached 60–70% confluence. Human fibroblasts were isolated from the same skin specimens used for explants after they were separated from the epithelium, and grown in fibroblast growth medium (FGM), which consists of Dulbecco's modified Eagle's medium, 0.6 mg/ml glutamine, and 10% fetal calf serum. Since the primary culture of dermal fibroblasts contained a small amount of keratinocytes, passages 3–5 were used for the experiment.

Measurement of MMP mRNA expression by real-time PCR

Real-time reverse transcriptase polymerase chain reaction (real-time PCR) assays (32) using SYBR® Green chemistry (33,34) were performed with a sequence detection system (ABI PRISM® 7700; PE Biosystems, Foster City, CA, USA) to quantify the MMP-1, MMP-8, and MMP-13 mRNA expression.

The fibroblasts of the keloid group and normal group were seeded at the density of 5×10^6 cells on a 100-mm Petri dish in 10 ml of culture medium. Forty-eight hours after seeding, the culture medium of each dish was changed to medium containing 1 μmol/l tretinoin. Total RNA was obtained with RNeasy® Mini Kit (Qiagen, Hilden, Germany) as described previously (35), at 0, 6, 12, 24 and 48 h after the medium change. In order to eliminate any residual genomic DNA, RNase-Free DNase Set (Qiagen) was also used. The concentration of each RNA sample was measured with a spectrophotometer (V-530 UV/VIS; Jasco, Tokyo, Japan).

A reverse transcriptase reaction was performed using RNA PCR Kit (AMV Ver.2.1; TaKaRa, Tokyo, Japan). We added 5 μg RNA to 100 μl of reaction mixture (final concentrations 5 mmol/l $MgCl_2$, 1 mmol/l dNTP mixture, 1 U/μl RNase inhibitor, 0.125 μmol/l oligo dT-Adaptor primer, 10 mmol/l Tris-HCl, 50 mmol/l KCl, pH 8.3) containing 25 U AMV Reverse Transcriptase XL, which was incubated at 42°C for 30 min, followed by inactivation of the enzyme at 99°C for 5 min with Program Temp Control System PC-700 (Astec, Fukuoka, Japan). The control reaction was performed simultaneously with an otherwise identical reaction, but without reverse transcriptase.

Real-time PCR was performed on 96-well optical reaction plates (ABI PRISM; PE Biosystems). Sequences of each oligonucleotide primers are shown in Table 2. All PCR reaction mixtures contained 25 μl of *Taq*Man SYBR® Green PCR Master Mix (2×) (PE Biosystems,), 0.25 μl of forward primer (10 pmol/μl), 0.25 μl of reverse primer (10 pmol/μl), 4 μl of each diluted sample, and 20.5 μl of double distilled water per well. PCR amplification of the identical sample was performed with both specific primer pairs of the target MMP gene and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene on the same reaction plate. The PCR reaction comprised 40 cycles, consisting of denaturing at 95°C (15 s), then annealing/extension at 60°C (1 min). In order to eliminate the possibility of contamination of genomic DNA during extraction of total RNA, the RNA extract before reverse transcription was amplified in the same way as the control, and no amplification was detected.

Measurement of secreted MMP protein by enzyme-linked immunosorbent assay

The fibroblasts, derived from keloid tissue or normal skin, were seeded at the density of 5×10^6 cells on a 100-mm Petri dish in 10 ml of culture medium as described above. The culture medium

Table 1. Profiles of skin samples used in the experiments

	Control group (normal skin)	Keloid group
Number	12	12
Age (years) (mean ± SD)	17–51 (32.8 ± 9.5)	8–58 (29.0 ± 15.9)
Sex (M/F)	6/6	5/7
Body sites	Scapular region (9), upper arm (2), shoulder (1)	Scapular region (4), upper arm (2) shoulder (2) chest (2), forearm (1), ear (1)

No significant difference was observed between the control and the keloid group ($P = 0.4907$).

Table 2. Oligonucleotide primers used in the real-time PCR amplification of MMPs

Gene	GenBank accession number	Primer sequence[1]	Nucleotide coordinates
Human MMP-1	X05231	ACGGATACCCCAAGGACATCT	1139–1159
		CTCAGAAAGAGCAGCATCGATATG	1192–1215
Human MMP-8	J05556	ACCAAAGAGATCACGGTGACAA	559–580
		TGAGCATCTCCTCCAATACCTTG	633–655
Human MMP-13	X75308	CCTGGAGCACTCATGTTTCCTAT	710–732
		GACTGGATCCCTTGTACATCGTC	773–795
Human GAPDH[2]	NM_17701	GAAGGTGAAGGTCGGAGTC	81–99
		GAAGATGGTGATGGGATTTC	287–306

[1]All primer sequences are written from 5' to 3'. For each primer pair, the top sequence is sense and the bottom sequence is antisense. [2]GAPDH is human glyceraldehyde-3-phosphate dehydrogenase and was used as a housekeeping gene (i.e. a control).

of each dish of the experimental group was changed to a medium containing 1 μmol/l tretinoin (with 10 μl of ethanol as a vehicle), or to that containing only 10 μl vehicle, at 48 h after seeding. Before assay, 2 ml of each culture supernatant was concentrated by freeze-drying (Freeze Dryer FRD-mini; Asahi Technoglass, Tokyo, Japan). Freeze-dried supernatants were dissolved in the assay buffer for the enzyme-linked immunosorbent assay (ELISA) system (0.03 mol/l H_3PO_4, 0.1 mol/l NaCl, 1% bovine serum albumin, 0.01 mol/l EDTA). For the MMP-1 assay, 10× concentrated samples were prepared, and for MMP-8 and MMP-13, 20× concentrated samples were prepared.

The culture supernatant of each dish was collected 96 h after the medium change. The BIOTRAK ELISA MMP-1, MMP-8, and MMP-13 System (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) was used for measurement of MMP-1, MMP-8, and MMP-13 protein levels in each culture supernatant, respectively. Standard and concentrated (10×) samples were incubated in microtiter wells precoated with a primary mouse antihuman MMP-1 monoclonal antibody followed by a secondary rabbit antihuman MMP-1 polyclonal antibody. The resulting antigen–antibody complex was detected using horseradish peroxidase-labeled donkey antirabbit IgG, and the conjugate quantified by a colorimetric reaction with 3,3′,5,5′-tetramethylbenzidine substrate. After stopping the reaction with 100 μl of 1 mol/l sulphilic acid, the resultant color was read at 450 nm with a microplate reader (Model 550; Bio-Rad Laboratories, Hercules, CA, USA). All samples were assayed in duplicate, and the concentration of the target protein in each sample was determined by interpolation from the standard curve.

Statistical analysis

All data are presented as mean ± standard error. The data were statistically analyzed using Student's *t*-test. Differences in the keloid and normal groups were tested using a paired *t*-test. Differences between the keloid and control groups were tested using an unpaired *t*-test. $P < 0.05$ was considered significant.

Results

MMP mRNA expression in keloid-derived fibroblasts and normal-skin-derived fibroblasts

MMP-1, MMP-8, and MMP-13 mRNA expression in the keloid group and the control group were measured by real-time PCR system, and the results are demonstrated in Fig. 1.

The normalized MMP-1 mRNA expression (MMP-1/GAPDH) was significantly downregulated in keloid-derived compared with normal fibroblasts ($P = 0.0001$), and the fold change vs. the average of the control group was 0.32 ± 0.02 (mean ± standard error). Similarly, the normalized MMP-8 mRNA expression was significantly downregulated in keloid-derived fibloblasts ($P = 0.0120$); the fold change vs. the average of the control group was 0.29 ± 0.02. However, the normalized MMP-13 mRNA expression was significantly elevated in keloid-derived fibroblasts ($P < 0.0001$); the fold change vs. the average of the control group was 21.21 ± 1.24. No age-related difference was observed in MMP expression in either normal or keloid-derived fibroblasts in this study using non-ultraviolet (UV)-exposed skin.

Effects of tretinoin on MMP mRNA expression in keloid-derived fibroblasts and normal-skin-derived fibroblasts.

Effects of tretinoin on MMP-1, MMP-8, and MMP-13 mRNA expression over time were also examined by real-time PCR, and the results are shown in Fig. 2. MMP-1 and MMP-8 mRNA expression in the control group were significantly upregulated, with the peak at 12 h after addition of tretinoin (2.03 ± 0.03 and 250.80 ± 4.98, respectively) ($P < 0.0001$), whereas no significant change was observed in the keloid group within 24 h after the addition of tretinoin. In contrast, markedly elevated MMP-13 mRNA expression in the keloid group was significantly suppressed by tretinoin with the peak suppression at 12 h (1.29 ± 0.04) ($P = 0.0003$). MMP-13 mRNA expression in the control group was not significantly changed by treatment with tretinoin.

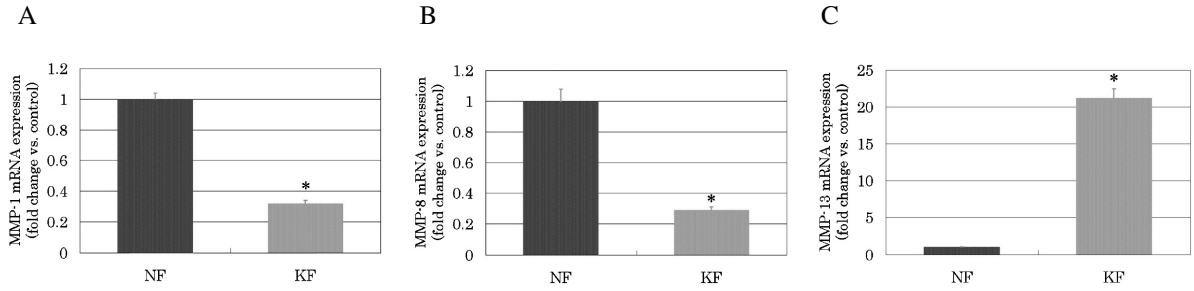

Figure 1. MMP-1, -8, and -13 (A, B, and C) mRNA expression in keloid-derived fibroblasts and normal dermal fibroblasts, quantified by real-time PCR. Normalized MMP mRNA expression were calculated as MMP mRNA/GAPDH mRNA. The values of the keloid group were presented as the fold change in normalized MMP mRNA expression relative to the average value of the control group (normal dermal fibroblasts). Values are means ± SE. * $P < 0.05$ vs. normal dermal fibroblasts. NF: normal dermal fibroblasts ($n = 12$), KF: keloid-derived fibroblasts ($n = 12$).

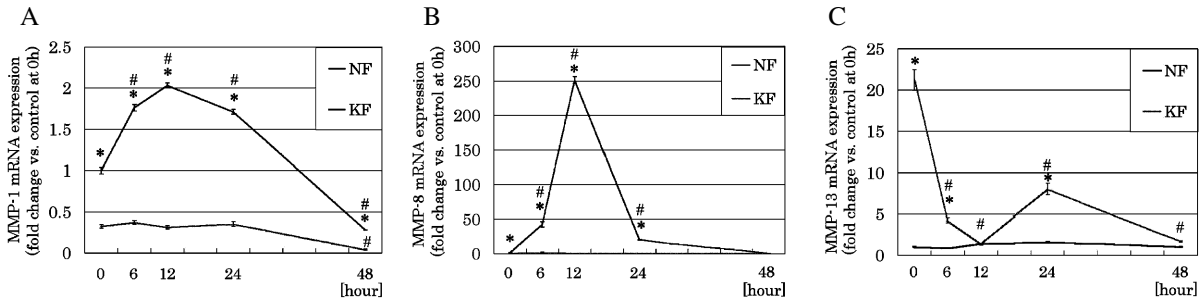

Figure 2. Sequential changes in MMP-1, -8, and -13 (A, B, and C) mRNA expression in keloid-derived fibroblasts and normal dermal fibroblasts quantified by real-time PCR. Normalized MMPs mRNA expression were calculated as MMP mRNA/GAPDH mRNA. The values of the keloid group were presented as the fold change in normalized MMP mRNA expression relative to the average value of the control group (normal dermal fibroblasts) at 0 h. Values are means ± SE. * $P < 0.05$ vs. normal dermal fibroblasts at each time point. # $P < 0.05$ vs. before treatment with tretinoin. NF: normal dermal fibroblasts ($n = 12$), KF: keloid-derived fibroblasts ($n = 12$).

MMP protein levels in the culture supernatants and effects of tretinoin on them

MMP-1, MMP-8, and MMP-13 protein levels in the culture supernatants in the keloid and control groups, and effects of tretinoin on these, were examined by ELISA. The results are shown in Fig. 3.

MMP-1 protein expression was significantly lower in the keloid (1.04 ± 0.03 ng/ml) than in the control group (6.16 ± 0.10 ng/ml) ($P < 0.0001$). Similarly, the MMP-8 protein level was significantly lower in the keloid (11.54 ± 0.24 pg/ml) than in the control group (15.36 ± 0.29 pg/ml) ($P = 0.0043$). However, the MMP-13 protein level was significantly elevated in the keloid group (17.53 ± 0.33 pg/ml) in contrast to the control group (6.71 ± 0.10 pg/ml) ($P < 0.0001$).

In both groups, tretinoin treatment for 96 h significantly elevated the MMP-1 (3.35 ± 0.07 ng/ml, 8.22 ± 0.09 ng/ml; $P < 0.0001$, $P = 0.0019$) and MMP-8 (21.21 ± 0.22 pg/ml, 30.13 ± 0.37 pg/ml; $P < 0.0001$ for both) protein levels. However, the markedly elevated MMP-13 protein level in the keloid group was significantly decreased after treatment with tretinoin for 96 h (8.56 ± 0.20 pg/ml) ($P < 0.0001$, while the MMP-13 protein level in the control group was modestly suppressed by tretinoin (6.23 ± 0.08 pg/ml) ($P = 0.0415$).

Discussion

MMP-1, MMP-8, and MMP-13 all degrade type I and type III collagen. Among the three MMPs, MMP-1 and MMP-8 most effectively degrade type III and type I collagen, respectively. The decrease in MMP-1 and MMP-8 may partly contribute to the accumulation of type I and type III collagen in keloid tissues, and this mechanism may be modulated by molecular interaction with MMP-13.

MMP-13 is an abnormal collagenase subtype that has been found in the bottom of chronic ulcers, where angiogenesis and fibrosis occur (36). On the other hand, MMP-1 and MMP-8 are considered to be 'normal' collagenase subtypes, which appear in normal wound-healing process (12,37). Before the discovery of MMP-13, reports had shown rather conflicting results concerning the collagenase activity in degradation of type I or total collagen in keloid tissues; some reports had shown normal (8), or decreased (9), and others showed increased (10) activity of collagenase.

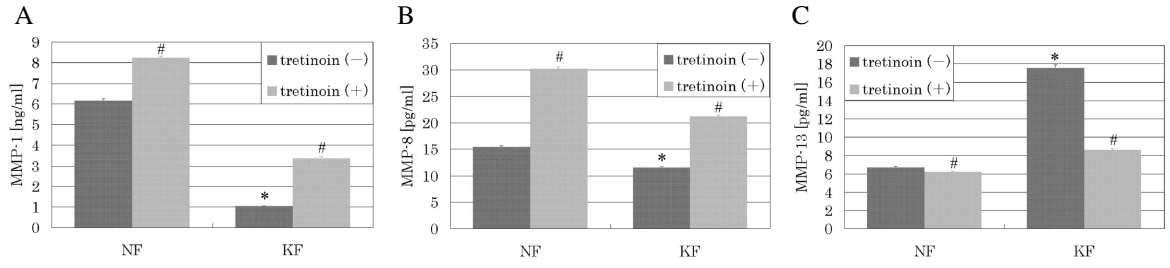

Figure 3. MMP-1, -8, and -13 (A, B, and C) protein levels in the culture supernatants of the keloid group and the control group measured by ELISA. The effects of tretinoin was examined after treatment of 1 μmol/l tretinoin for 96 h. Values are means ± SE. * $P < 0.05$ vs. the control group (without addition of tretinoin). # $P < 0.05$ vs. before treatment with tretinoin. NF: normal dermal fibroblasts ($n = 12$), KF: keloid-derived fibroblasts ($n = 12$).

These variable results may be partly due to different areas of keloid tissue, for example, a marginal or a central area, being used. In our preliminary study, MMP-13 mRNA expression was found to be markedly higher in marginal than central areas of keloid tissues (data not shown). In the present study, comparison of MMP expression was performed using a marginal area of each keloid sample.

Our study has demonstrated a significant increase in MMP-13 expression and a decrease in expression of MMP-1 and MMP-8 in keloid-derived fibroblasts, in both mRNA and protein levels. The remodeling of the surrounding matrix by MMP-13 may interfere in the normal degradation process of wound healing in keloid tissues, and may initiate the negative feedback mechanism to transcription of MMP-1 and MMP-8, which act in the normal wound-healing process. These mechanisms could be related to the chronic inflammation and infiltration into circumferential normal skin seen in keloid tissues.

To correct the abnormal wound-healing mechanism mentioned above, we assumed that retinoids are potent additives, and then investigated the influences of tretinoin on abnormal MMP expression of keloid tissues. The present study revealed that addition of tretinoin to the culture media caused significant downregulation of MMP-13 in keloid-derived fibroblasts at both mRNA and protein levels, and significant upregulation of MMP-8 in normal dermal fibroblasts. Although mRNA expression of MMP-1 was not clearly affected in the keloid-derived fibroblasts after treatment with tretinoin, upregulation of MMP-1 and MMP-8, and downregulation of MMP-13 at the protein level, may suggest that tretinoin reverses the specific changes in the MMP expression profile of keloids. We also examined mRNA expression of four subtypes of TIMP (TIMP-1, -2, -3, and -4); all of these subtypes were upregulated in keloid-derived fibroblasts, but we did not detect any significant changes after treatment with tretinoin (data not shown).

A small number of previous studies reported effects of retinoids on primary cultured human dermal fibroblasts. Daly et al. (38) demonstrated that tretinoin significantly reduces collagen production of human primary cultured fibroblasts. Abergel et al. (39) reported that tretinoin and isotretinoin significantly inhibit degradation activity of type I collagen fibers in keloid-derived fibroblasts. On the other hand, in the field of cancer cell study, degradation of type I and type IV collagen, and invasion into collagen matrix was reported to be significantly inhibited by retinoids (28). The results of our study and those in the literature suggest that a remarkable inhibition of degradation of type I collagen by tretinoin is presumably due to a strong inhibition of MMP-13 expression by tretinoin, which negates the upregulation of MMP-8.

A previous study using human skin *in vivo* suggested that MMP-1, but not MMP-13 were upregulated in human keratinocytes and fibroblasts by UV irradiation, MMP-8 was upregulated in human neutrophils by UV, and only MMP-1 upregulated by UV was blocked by tretinoin (22). Our *in vitro* study using fibroblasts taken from non-UV-exposed skin (keloid-prone location) showed that only MMP-13 was significantly suppressed by tretinoin.

Expression of MMP-1 and MMP-13 are known to be induced at the transcriptional level by a variety of growth factors (14), and these extracellular stimuli result in activation of the nuclear AP-1 trascription factor complex, which binds to the AP-1 *cis*-regulatory element in the promoter region of the relevant *MMP* gene and potently activates its transcription (15). This AP-1-dependent activation of inducible MMPs is potently inhibited by glucocorticoids (17) and tretinoin (18) at the transcriptional level. The present results reveal that MMP-13 is upregulated in keloid-derived fibroblasts, and that this upregulation of MMP-13 is inhibited at the transcriptional level by tretinoin, suggesting that this upregulation in keloids is induced via the AP-1 pathway. However, exactly how tretinoin upregulates MMP-1 and MMP-8 in keloid-derived fibroblasts and in normal dermal fibroblasts remains unknown. Further investigations of the regulatory mechanisms are necessary.

Conclusion

In this study, it is suggested that MMPs are abnormally regulated in keloid tissues and chronic ulcers, and that these abnormal changes may be reversed by treatment with retinoids. Tretinoin may improve the chronic inflammation seen in keloids and prevent expansion of keloid tissues into circumferential normal skin.

Since 1999, we have been performing clinical trials with tretinoin aqueous gel (0.1–0.4%) for treatment of keloids. Our preliminary results demonstrate that topical application of tretinoin on keloids has unique advantages. In most cases, itching and/or tenderness of the lesions disappeared after topical tretinoin (in preparation), although the volume-suppressing effects on the fibrosis were quite modest. We assume that the

effects of tretinoin on MMP expression resulted in suppression of chronic inflammation, and prevention of growth and invasion of keloid tissues. Considering the limited clinical improvements and side-effects generated by existing techniques, the clinical use of topical tretinoin looks promising. Thus, the molecular mechanisms of the regulation of MMPs deserve further investigation. The results of this study may be helpful in developing more chemically stable synthesized retinoids, which specifically reverse the abnormal expression of MMPs and prevent cell growth in keloids, with minimum side-effects.

Acknowledgements

This study was supported in part by Grants-in-Aid for Fundamental Research A(2) 12307039 and B(2) 12470374 from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

References

1. Tredget E E, Nedelec B, Scott P G, Ghahary A. Hypertrophic scars, keloids, and contractures. The cellular and molecular basis for therapy. Surg Clin North Am 1997: 77: 701–730.
2. Russell J D, Witt W S. Cell size and growth characteristics of cultured fibroblasts isolated from normal and keloid tissue. Plast Reconstr Surg 1976: 57: 207–212.
3. Diegelmann R F, Rothkopf L C, Cohen I K. Measurement of collagen biosynthesis during wound healing. J Surg Res 1975: 19: 239–243.
4. Horslev-Petersen K, Kim K Y, Pedersen L R et al. Serum aminoterminal type III procollagen peptide. Relation to biosynthesis of collagen type III in experimentally induced granulation tissue in rats. APMIS 1988: 96: 793–804.
5. Bailey A J, Bazin S, Sims T J, Le Lous M, Nicoletis C, Delaunay A. Characterization of the collagen of human hypertrophic and normal scars. Biochim Biophys Acta 1975: 405: 412–421.
6. Uitto J, Perejda A J, Abergel R P, Chu M L, Ramirez F. Altered steady-state ratio of type I/III procollagen mRNAs correlates with selectively increased type I procollagen biosynthesis in cultured keloid fibroblasts. Proc Natl Acad Sci USA 1985: 82: 5935–5939.
7. Peltonen J, Hsiao L L, Jaakkola S et al. Activation of collagen gene expression in keloids: co-localization of type I and VI collagen and transforming growth factor-beta 1 mRNA. J Invest Dermatol 1991: 97: 240–248.
8. McCoy B J, Cohen I K. Collagenase in keloid biopsies and fibroblasts. Connect Tissue Res 1982: 9: 181–185.
9. Abergel R P, Pizzurro D, Meeker C A et al. Biochemical composition of the connective tissue in keloids and analysis of collagen metabolism in keloid fibroblast cultures. J Invest Dermatol 1985: 84: 384–390.
10. Cohen I K, Dieglemann R F, Keiser H R. Collagen metabolism in keloid and hypertrophic scar. In: Longacre JJ, ed. The Ultrastructure of Collagen. Springfield, IL: Charles C Thomas, 1976: 199–212.
11. Kahari V M, Saarialho-Kere U. Matrix metalloproteinases in skin. Exp Dermatol 1997: 6: 199–213.
12. Nwomeh B C, Liang H X, Diegelmann R F, Cohen I K, Yager D R. Dynamics of the matrix metalloproteinases MMP-1 and MMP-8 in acute open human dermal wounds. Wound Rep Reg 1998: 6: 127–134.
13. Freije J M, Diez-Itza I, Balbin M et al. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. J Biol Chem 1994: 269: 16766–16773.
14. Mauvel A. Cytokine regulation of metalloproteinase gene expression. J Cell Biochem 1993: 53: 288–295.
15. Gaire M, Magbauna Z, McDonnel S, McNeil L, Lovett DH, Matrisian LM. Structure and expression of the human gene for matrix metalloproteinase matrilysin. J Biol Chem 1994: 269: 2032–2040.
16. Karin M, Liu Z, Zandi E. AP-1 function and regulation. Curr Opin Cell Biol 1997: 9: 240–246.
17. Mauvel A, Halcin C, Vasiloudes P, Parks W C, Kurkinen M, Uitto J. Uncoordinate regulation of collagenase, stromelysin, and tissue inhibitor of metalloproteinases genes by prostaglandin E2: selective enhancement of collagenase gene expression in human dermal fibroblasts in culture. J Cell Biochem 1994: 54: 465–472.
18. Nicholson R C, Mader S, Nagpal S, Leid M, Rochette-Egly C, Chambon P. Negative regulation of rat stromelysin gene promotor by retinoic acid is mediated by an AP-1 binding site. EMBO J 1990: 9: 4443–4454.
19. Van Wart H E, Birkedal-Hansen H. The cysteine switch. A principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci 1990: 87: 5578–5582.
20. Gomez D E, Alonso D F, Yoshiji H, Thorgeirsson U P. Tissue inhibitors of metalloproteinases: structure, regulation, and biological functions. Eur J Cell Biol 1997: 74: 111–122.
21. Fisher G J, Wang Z Q, Datta S C, Varani J, Kang S, Voorhees JJ. Pathophysiology of premature skin aging induced by ultraviolet light. N Engl J Med 1997: 13: 1419–1428.
22. Fisher GJ, Choi HC, Beta-Csorgo Z et al. Ultraviolet irradiation increases matrix metalloptoteinases-8 protein in human skin in vivo. J Invest Dermatol 2001: 117: 219–226.
23. Kang S, Fischer G J, Vorhees J J. Photoaging and topical tretinoin: therapy, pathogenesis, and prevention. Arch Dermatol 1997: 133: 1280–1284.
24. MacDougall J R, Matrisian L M. Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. Cancer Metast Rev 1995: 14: 351–362.
25. Chambers A F, Matrisian L M. Changing views of the role of matrix metalloproteinases in metastasis. J Natl Cancer Inst 1997: 89: 1260–1270.
26. Lippman S M, Meyskens F L. Result of the use of vitamin A and retinoids in cutaneous malignancies. Pharmac Ther 1989: 40: 107–122.
27. Hansen L A, Sigman C C, Andreola F, Ross S A, De Kelloff G J, Luca L M. Retinoids in chemoprevention and differentiation therapy. Carcinogenesis 2000: 21: 1271–1279.
28. Schoenermark M P, Mitchell T I, Rutter J L, Reczek P R, Brinckerhoff C E. Retinoid-mediated suppression of tumor invasion and matrix metalloproteinase synthesis. Ann NY Acad Sci 1999: 878: 466–486.

29. Ueda K, Furuya E, Yasuda Y, Oba S, Tajima S. Keloids have continuous high metabolic activity. Plast Reconstr Surg 1999: 104: 694–698.
30. Hunt T K. Basic principles of wound healing. J Trauma 1990: 30: S122–S128.
31. Martin G M, Tuan A A. Definitive cloning technique for human fibroblast cultures. Exp Biol Med 1966: 123: 138–140.
32. Cane P A, Cook P, Ratcliffe D, Mutimer D, Pillay D. Use of real-time PCR and fluorimetry to detect lamivudine resistance-associated mutations in hepatitis B virus. Antimicrob Agents Chem 1999: 43: 1600–1608.
33. Schmittgen T D, Zakrajsek B A, Mills A G, Gorn V, Singer M J, Reed M W. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay. comparison of endpoint and real-time methods. Anal Biochem 2000: 285: 194–204.
34. Yin J L, Shackel N A, Zekry A et al. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. Immunol Cell Biol 2001: 79: 213–221.
35. Schafer H, Trauzold A, Siegel E G, Folsch U R, Schmidt W E et al. PRG1: a novel early-response gene transcriptionally induced by pituitary adenylate cyclase activating polypeptide in a pancreatic carcinoma cell line. Cancer Res 1996: 56: 2641–2648.
36. Vaalamo M, Mattila L, Johansson N et al. Distinct populations of stromal cells express collagenase-3 (MMP-13) and collagenase-1 (MMP-1) in chronic ulcers but not in normally healing wounds. J Invest Dermatol 1997: 109: 96–101.
37. Saarialho-Kere U K. Patterns of matrix metalloproteinase and TIMP expression in chronic ulcers. Arch Dermatol Res 1998: 290: S47–S54.
38. Daly T J, Weston W L. Retinoid effects on fibroblast proliferation and collagen synthesis in vitro and on fibrotic disease in vivo. J Am Acad Dermatol 1986: 15: 900–902.
39. Abergel R P, Meeker C A, Oikarinen H, Oikarinen A I, Uitto J. Retinoid modulation of connective tissue metabolism in keloid fibroblast cultures. Arch Dermatol 1985: 121: 632–635.