

# Matrix Metalloproteinases of Epithelial Origin in Facial Sebum of Patients with Acne and their Regulation by Isotretinoin

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**Acne vulgaris is a skin disorder of the sebaceous follicles, involving hyperkeratinization and perifollicular inflammation. Matrix metalloproteinases (MMP) have a predominant role in inflammatory matrix remodeling and hyperproliferative skin disorders. We investigated the expression of MMP and tissue inhibitors of MMP (TIMP) in facial sebum specimens from acne patients, before and after treatment with isotretinoin. Gelatin zymography and Western-blot analysis revealed that sebum contains proMMP-9, which was decreased following *per os* or topical treatment with isotretinoin and in parallel to the clinical improvement of acne. Sebum also contains MMP-1, MMP-13, TIMP-1, and TIMP-2, as assessed by ELISA and western blot, but only MMP-13 was decreased following treatment with isotretinoin. The origin of MMP and TIMP in sebum is attributed to keratinocytes and sebocytes, since we found that HaCaT keratinocytes in culture secrete proMMP-2, proMMP-9, MMP-1, MMP-13, TIMP-1, and TIMP-2. SZ95 sebocytes in culture secreted proMMP-2 and proMMP-9, which was also confirmed by microarray analysis. Isotretinoin inhibited the arachidonic acid-induced secretion and mRNA expression of proMMP-2 and -9 in both cell types and of MMP-13 in HaCaT keratinocytes. These data indicate that MMP and TIMP of epithelial origin may be involved in acne pathogenesis, and that isotretinoin-induced reduction in MMP-9 and -13 may contribute to the therapeutic effects of the agent in acne.**

Key words: acne/isotretinoin/keratinocytes/MMP/sebocytes/TIMP  
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Acne vulgaris is the most common skin disorder, initiated due to sebaceous gland hyperactivity and hyperseborrhea, abnormal hyperproliferation of ductal keratinocytes and keratinization of the acroinfundibular epithelium of the sebaceous follicle, and inflammatory signaling giving rise to microcomedones (Zouboulis, 2001a). Sebum consists principally of triglycerides, which are subsequently hydrolyzed to yield free fatty acids and glycerol, and in addition sebum also contains keratinocytes, microorganisms, neutrophils, and macrophages (Toyoda and Morohashi, 2001). Although individual acne lesions spontaneously regress, persistent cases of acne vulgaris often require pharmacological intervention.

Pharmacotherapy of acne vulgaris includes a variety of compounds, with the retinoids possessing a prevailing position (Zouboulis, 2001c). Among the retinoic acid (RA) analogues that have been established for systemic and/or

topical treatment of hyperproliferative skin disorders, inflammatory diseases, and cancer (Orfanos *et al*, 1997; Zouboulis, 2001c), the non-aromatic retinoid isotretinoin (13-*cis*-RA) secures effective treatment of acne, but it may also cause adverse and toxic effects (Zouboulis and Orfanos, 2000). The antiproliferative effect of isotretinoin on sebocytes is manifested through its isomerization into all-*trans*-RA and binding to nuclear RA receptors (Zouboulis, 2001b; Tsukada *et al*, 2002). Isotretinoin displays key regulatory functions on epidermal growth and differentiation (Fisher and Voorhees, 1996) but the cellular and biochemical alterations associated with them are not fully clarified.

Isotretinoin has been reported to affect matrix metalloproteinases (MMP) (Jimenez *et al*, 2001; Zhu *et al*, 2001; Devy *et al*, 2002), a family of Zn-dependent metalloproteinases that has been implicated in skin biology during inflammatory matrix remodeling, neovascularization, wound healing, and malignant transformation. Thus, it appears that MMP have a predominant role in pathological manifestations of diseases treated with retinoids, such as hyperproliferative skin disorders, inflammatory diseases, and cancer (Orfanos *et al*, 1997). MMP degrade extracellular matrix molecules during physiological and pathological tissue remodeling (Visse and Nagase, 2003). The MMP gene

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Abbreviations: aRNA, amplified RNA; cDNA, complementary DNA; MMP, matrix metalloproteinases; RA, retinoic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TIMP, tissue inhibitors of metalloproteinases

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family encodes for several proenzymes (proMMP) with common and distinctive structural and functional properties, classified as: (a) collagenases 1–4 (MMP-1, -8, -13 and -18, respectively), (b) gelatinases A and B (MMP-2 and -9, respectively), (c) stromelysins 1–3 (MMP-3, -10, and -11, respectively), (d) matrilysins 1–2 (MMP-7 and -26, respectively), (e) membrane type 1–6 (MT-MMP: MMP-14, -15, -16, -17, -24, and -25, respectively), and (f) various others (MMP-12, -19, -20, -21, -23, -27, and -28) (Visse and Nagase, 2003). The activity of MMP is tightly regulated during proMMP expression (Stetler-Stevenson *et al*, 1993) and by proteolytic activation of secreted latent MMP (Cao *et al*, 1995). MMP, both in latent and activated form, are also regulated by the formation of stoichiometric 1:1 complexes with specific inhibitors, known as tissue inhibitors of metalloproteinases (TIMP) (Stetler-Stevenson *et al*, 1993). Four TIMP have been identified, but inactivation of MMP by TIMP is predominantly attributed to TIMP-1 and -2. TIMP-1 forms high-affinity, non-covalent complexes with latent MMP-9 and active MMP-1, -3, and -9, whereas TIMP-2 forms complexes with latent and active MMP-2 (Gomez *et al*, 1997). TIMP-1/proMMP-9 and TIMP-2/proMMP-2 complexes are also capable of inhibiting active MMP through the unoccupied N-terminal domain of the inhibitor in the complex (Itoh *et al*, 1995).

The effect of isotretinoin on MMP, however, is contradictory, since there is evidence suggesting that retinoids increase (Jimenez *et al*, 2001), reduce (Neuville *et al*, 1999; Leville *et al*, 2000; Axel *et al*, 2001; Frankenberger *et al*, 2001; Tsang and Crowe, 2001; Devy *et al*, 2002; Osteen *et al*, 2002), or do not affect (Zhu *et al*, 2001) the activity or gene expression of MMP in various human and animal biological systems and cell lines. Furthermore, there is no report concerning the presence of MMP in lesions of acne vulgaris or the effect of isotretinoin treatment on MMP in sebum of acne patients.

In this study, we investigated the presence of gelatinases, collagenases, and TIMP in sebum samples of facial lesions from patients with acne vulgaris, as well as in cultures of HaCaT keratinocytes and SZ95 sebocytes, and the effect of isotretinoin in these parameters. We found that proMMP-9, MMP-1, MMP-13, and TIMP-1 and TIMP-2 are present in the sebum of facial lesions from acne patients, possibly originating from keratinocytes and sebocytes, and that MMP-9 and -13 are reduced in parallel to treatment with isotretinoin and the clinical improvement of the lesions.

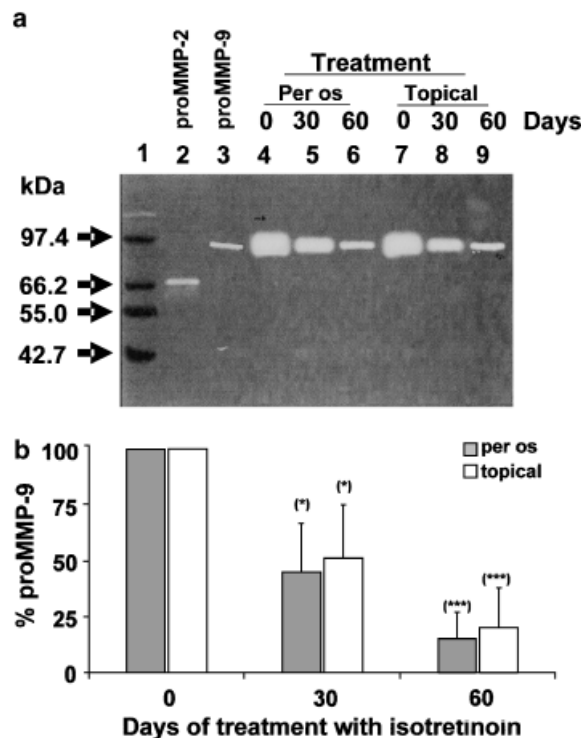
## Results

### Acne sebum samples

**Keratinocyte and bacteriologic findings** Examination of sebum specimens, diluted to contain the same amount of protein per mL, revealed the presence of keratinocytes under a light microscope in all 0 d samples from 59 patients. After 60 d of topical (0.05% once daily) or systemic (1 mg per kg per d *per os*) treatment with isotretinoin, examination of 60 d sebum samples showed that keratinocytes were present only in 11 of 59 patients and in considerably diminished numbers. Bacterial cultures of sebum specimens

revealed the presence of *P. acnes* (propionibacterium acnes) in all 0 d samples and of *Staphylococcus epidermidis* in two samples. No other microorganisms were detected in 0 d samples. Furthermore, no bacteria could be detected in any of the 60 d samples.

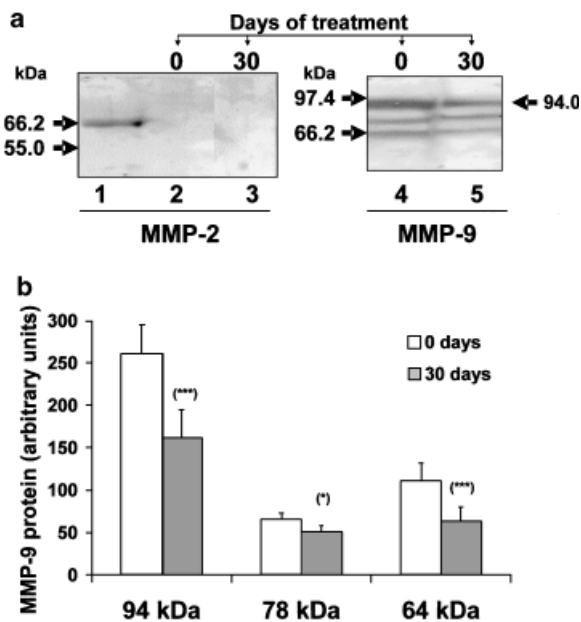
**Gelatinase activity** Gelatin zymography analysis revealed that 0 d sebum samples of facial acne lesions express gelatinase activity, which produced major lysis bands (Fig 1a), which comigrated as purified proMMP-9 (92.0 kDa). Gelatinolytic activity was completely inhibited by developing the zymograms in the presence of the metal chelators Na<sub>2</sub>EDTA (20 mM) or 1,10-phenanthroline (4 mM), but was unaffected by *N*-ethyl-maleimide (5 mM) (data not shown), indicative of a metalloproteinase, corresponding to proMMP-9. Zymography analysis of samples obtained at 30 and 60 d following systemic *per os* or topical treatment with isotretinoin (Fig 1a), indicated that gelatinolytic activity was reduced in relation to the duration of treatment. Quantification of zymography lysis bands of all acne samples tested, using a computer-assisted image analysis program (Fig 1b), revealed that the decrease in gelatinolytic activity was statis-



**Figure 1**  
**Gelatinase activity is reduced in sebum of facial acne lesions in a time-dependent manner during therapy with isotretinoin.** (a) Representative gelatin zymography in aliquots of sebum (5 µg of protein) during systemic *per os* (lanes 4–6) and topical (lanes 7–9) treatment with isotretinoin. Arrows indicated pre-stained standard protein molecular weight markers (lane 1): phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), L-glutamic dehydrogenase (55.0 kDa), and ovalbumin (42.7 kDa). Lane 2: migration of purified promatrix metalloproteinase (proMMP)-2 (72 kDa); lane 3: proMMP-9 (92 kDa); lanes 4 and 7: 0 d samples; lanes 5 and 8: 30 d samples; lanes 6 and 9: 60 d samples. (b) Quantitative analysis of gelatinolytic activity in sebum following systemic *per os* (n=23) or topical (n=36) treatment of acne patients with isotretinoin using a computer-supported image analysis program. Each bar represents the mean ± SD from n specimens. Statistical significance: \*p<0.05; \*\*\*p<0.01 as compared with 0 d samples, which were regarded as 100% gelatinolytic activity.

tically significant at 30 d ( $p < 0.05$ ) and 60 d ( $p < 0.01$ ) of treatment, whereas the route of administration of isotretinoin did not influence the decrease in gelatinase activity.

**Detection of gelatinases and effect of isotretinoin treatment** Using human MMP-2 antiserum, we found that there was no immunoreactivity for MMP-2 in 0 and 30 d sebum samples (Fig 2a, lanes 2 and 3). Using human MMP-9 antiserum, three distinct immunoreactive bands with  $M_r$  94.0, 78.0, and 64.0 kDa were revealed in 0 d sebum samples by western blotting (Fig 2a, lane 4). The upper band corresponds to proMMP-9 and comigrated as reference sample of proMMP-9 (94.0 kDa) that has been previously fully characterized from human periprosthetic tissue of loose hip endoprostheses (Syggelos *et al*, 2001). The two lower immunoreactive bands may be attributed to endogenous activation or degradation of the 94.0 kDa proenzyme, due to the experimental conditions adopted (Kerkela and Sarialho-Kere, 2003). The fact that the 78.0 and 64.0 kDa proteins identified by western blotting could not be visualized by gelatin zymography indicates that these proteins do not possess gelatinolytic activity. Treatment with isotretinoin for 30 d resulted in reduced expression of all the above-



**Figure 2**  
**Western blot analysis of gelatinases in sebum samples indicates the presence of matrix metalloproteinase (MMP)-9 but not of MMP-2.** (a) Representative western blot analysis of sebum during systemic *per os* treatment with isotretinoin. Analysis was performed using human MMP-2 antiserum: lane 1: purified promatrix metalloproteinase (proMMP)-2 (72.0 kDa), lane 2, 0 d sample; lane 3, 30 d sample, and human MMP-9 antiserum: lane 4, 0 d sample; lane 5, 30 d sample. Arrow on the right indicates the reference sample of proMMP-9 (94.0 kDa), which has been previously fully characterized from human periprosthetic tissue of loose hip endoprostheses. Arrows on the left indicate molecular weight size markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; L-glutamic dehydrogenase, 55.0 kDa). (b) Quantitative analysis of western blots of MMP-9 immunoreactive bands of sebum following systemic *per os* treatment of acne patients ( $n = 23$ ) with isotretinoin for 30 d, using a computer-supported image analysis program. Each bar represents the mean  $\pm$  SD from  $n$  specimens. Statistical significance: \* $p < 0.05$ ; \*\*\* $p < 0.01$  as compared with 0 d samples.

described bands of MMP-9 (Fig 2a, lane 5, *per os* treatment). Quantification of the chemiluminescence of each band using a computer-assisted image analysis program revealed that the observed reduction was statistically significant at 30 d of treatment ( $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.01$ , respectively, for each band) (Fig 2b), whereas the route of administration of isotretinoin did not influence the decrease in MMP-9 immunoreactivity (results not shown).

**Effect of isotretinoin on gelatinases in vitro** In order to investigate whether isotretinoin has a direct inhibitory effect on the activity of gelatinases, we subjected sebum samples (0 d, corresponding to 5  $\mu$ g protein) or commercially available proMMP-2 and -9 (1 ng) to gel electrophoresis, removed sodium dodecyl sulfate (SDS), and conducted gelatin lysis of the gels by incubating in enzyme buffer in the presence of isotretinoin (0–1 mM) for 18 h, at 37°C. Alternatively, we pre-incubated sebum samples (0 d, corresponding to 5  $\mu$ g protein) or commercially available proMMP-2 and -9 (1 ng), with isotretinoin (0–1 mM) for 30 min at room temperature, subjected samples to gel electrophoresis, removed SDS, and conducted to gelatin lysis of the gels by incubating in enzyme buffer for 18 h, at 37°C. Analysis of the results revealed that neither the presence of isotretinoin during gelatin zymography (results not shown) nor pre-treatment of the samples with isotretinoin for 30 min influenced proMMP-9 present in 0 d sebum samples (Fig 3a) or purified latent gelatinases (Fig 3b).

**Detection of collagenases by western blotting and effect of isotretinoin treatment** Using polyclonal antibodies for MMP-1, three distinct immunoreactive bands were revealed in 0 d sebum samples by western blotting (Fig 4a, lane 2), with  $M_r$  52.4, 45.0, and 43.5 kDa as estimated using molecular weight markers. These bands corresponded to purified human MMP standards: proMMP-1 (52.4 kDa) and fully active MMP-1 (45.0 and 43.5 kDa), respectively, in comparison with reference samples of proMMP-1 and MMP-1 (Fig 4a, lane 1), which have been previously fully characterized from human periprosthetic tissue of loose hip endoprostheses (Syggelos *et al*, 2001). The ratio of latent to active MMP-1 was approximately 8/1. Treatment with isotretinoin for 30 or 60 d did not affect the expression of latent or active MMP-1 (Fig 4a, lane 3, topical treatment, 30 d).

Using polyclonal antibodies for MMP-13, two distinct immunoreactive bands were revealed in 0 d sebum samples by western blotting (Fig 4b, lane 1), with  $M_r$  65.0 and 52.0 kDa as estimated using molecular weight markers. These bands corresponded to purified human MMP standards: proMMP-13 and fully active MMP-13, respectively, in comparison with reference samples of proMMP-13 and MMP-13 (Fig 4b, lane 3), which have been previously fully characterized from human periprosthetic tissue of loose hip endoprostheses (Syggelos *et al*, 2001). The ratio of latent to active MMP-13 was approximately 1/2. Treatment with isotretinoin for 30 or 60 d significantly reduced the expression of latent or active MMP-13 (Fig 4b, lane 3, topical treatment, 30 d).

**Determination of collagenases by ELISA and effect of isotretinoin treatment** Measurement of MMP-1 and -13 by

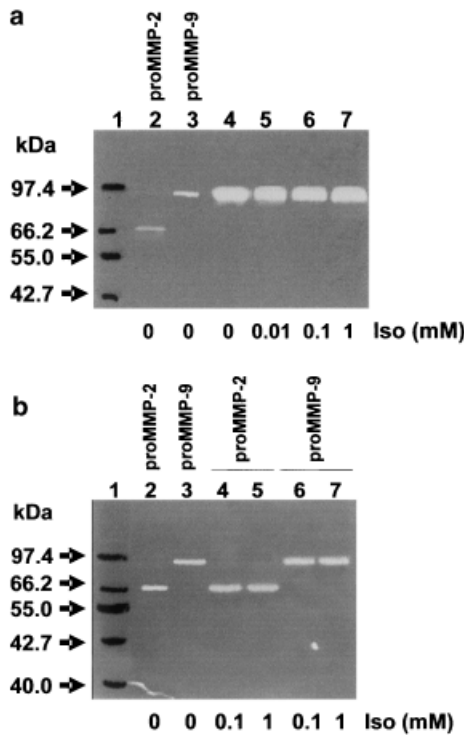


Figure 3

**Isotretinoin does not influence gelatinase *in vitro*.** (a) Sebum samples (0 d, corresponding to 5  $\mu$ g protein) were preincubated with isotretinoin (0–1 mM) for 30 min at room temperature and subjected to gelatin zymography, as described in Materials and Methods. Lane 4, 0 mM; lane 5, 0.01 mM; lane 6, 0.1 mM; lane 7, 1 mM. (b) Commercially available purified promatrix metalloproteinase (proMMP)-2 and proMMP-9 (1 ng protein) were treated as above. ProMMP-2: lane 4, 0.01 mM; lane 5, 1 mM. ProMMP-9: lane 6, 0.1 mM; lane 7, 1 mM, lane 7. Arrows and lane 1 indicate molecular weight markers: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), L-glutamic dehydrogenase (55.0 kDa), ovalbumin (42.7 kDa), and aldolase (40.0 kDa). Lane 2: purified proMMP-2 (72 kDa) and lane 3 purified proMMP-9 (92 kDa).

ELISA revealed that sebum samples expressed both collagenases (Table I, Fig 4c). In 0 d sebum samples of patients grouped for *per os* treatment, MMP-1 was  $105 \pm 20$  pg per  $\mu$ g of protein and MMP-13 was  $92 \pm 8$  pg per  $\mu$ g of protein. Similar values were obtained in 0 d sebum samples from patients grouped for topical administration. MMP-1 was not affected following treatment with isotretinoin for 60 d, irrespective of the route of administration. In contrast, MMP-13 was significantly reduced by about 55% and 58% after 30 and 60 d of *per os* treatment with isotretinoin, respectively ( $p < 0.02$ ). This effect was also evident after topical administration of isotretinoin, resulting in a significant decrease of MMP-13 by approximately 50% and 60% after 30 and 60 d of treatment with isotretinoin, respectively ( $p < 0.02$ ).

**Production of TIMP** Measurement of TIMP-1 and -2 by ELISA in 0 d sebum samples of patients grouped for *per os* administration indicated the presence of TIMP-1 ( $405 \pm 198$  pg per mg protein) and TIMP-2 ( $219 \pm 84$  pg per mg protein) (Fig 5). Similar values were obtained in 0 d sebum samples from patients grouped for topical administration. Treatment with isotretinoin did not influence the production of TIMP, irrespective of the route of administration (Fig 5).

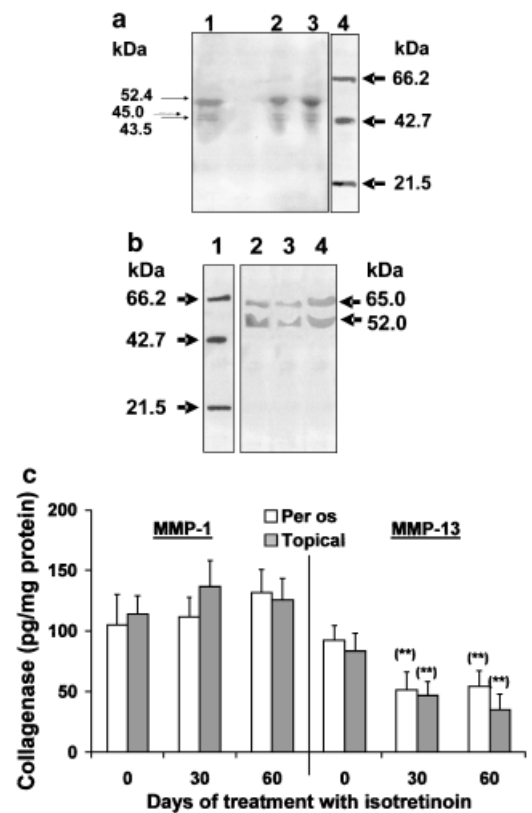


Figure 4

**Matrix metalloproteinase (MMP)-1 and MMP-13 are expressed in sebum of facial acne lesions, and MMP-13 expression is reduced during isotretinoin treatment.** (a) Representative western blot analysis of sebum during topical treatment with isotretinoin using polyclonal antibodies for MMP-1. Lane 1 and arrows on the left: immunoreactive bands from reference samples of promatrix metalloproteinase (proMMP)-1 (52.4 kDa) and MMP-1 (45.0 and 43.5 kDa), which have been fully characterized previously from human periprosthetic tissue of loose hip endoprostheses. Lane 2: immunoreactive bands of 0 d sebum samples. Lane 3: immunoreactive bands of 30 d sebum samples following topical treatment with isotretinoin. Lane 4 and arrows on the right indicate protein molecular weight markers: bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), and soybean trypsin inhibitor (21.5 kDa). (b) Representative western blot analysis of sebum during topical treatment with isotretinoin using polyclonal antibodies for MMP-13. Lane 1 and arrows on the left indicate protein molecular weight markers: bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa) and soybean trypsin inhibitor (21.5 kDa). Lane 2: immunoreactive bands of 0 d sebum samples. Lane 3: immunoreactive bands of 30 d sebum samples following topical treatment with isotretinoin. Lane 4 and arrows on the right: immunoreactive bands from reference samples of proMMP-13 (65.0 kDa) and MMP-13 (52.0 kDa) from human periprosthetic tissue. (c) Aliquots of sebum samples were assessed for MMP-1 and MMP-13 by ELISA, prior to and following *per os* ( $n = 23$ ) and topical ( $n = 36$ ) treatment of acne patients with isotretinoin, for 30 and 60 d. Each bar represents the mean  $\pm$  SD of triplicate determinations from  $n$  samples. Statistical significance: \*\* $p < 0.02$  as compared with 0 d sample.

**HaCaT keratinocyte and SZ95 sebocyte cultures** In order to elucidate the origin of MMP and TIMP in sebum samples, we examined their expression in HaCaT keratinocytes and SZ95 sebocyte cultures after 12, 24, and 48 h of incubation with or without arachidonic acid, in the presence and in the absence of isotretinoin. Arachidonic acid, a pro-inflammatory essential fatty acid, was included in the study since it has also been reported to induce sebaceous lipid synthesis (Wróbel *et al*, 2003) and may be involved in acne patho-

**Table I. Expression of collagenases, gelatinases, and TIMP in acne sebum, HaCaT keratinocytes, and SZ95 sebocytes as detected by gelatin zymography, western blotting, ELISA, and RT-PCR**

Nomenclature	Sebum of acne patients: proteins		HaCaT keratinocytes						SZ95 sebocytes					
			Proteins			mRNA			Proteins			mRNA		
	Untreated	Iso	Untreated	AA	Iso	Untreated	AA	Iso	Untreated	AA	Iso	Untreated	AA	Iso
Collagenases														
MMP-1	D	↔	D	↑	↔	D	↑	↔	ND	ND	ND	ND	ND	ND
MMP-13	D	↓	D	↔	↓ <sup>c</sup>	D	↑ <sup>c</sup>	↓	ND	ND	ND	ND	ND	ND
Gelatinases														
MMP-2 <sup>a</sup>	ND	ND	D	↑	↓ <sup>b</sup>	D	↑	↓ <sup>b,c</sup>	D	↑	↓ <sup>b</sup>	D	↑	↓ <sup>b,c</sup>
MMP-9 <sup>a</sup>	D	↓	D	↑	↓ <sup>b</sup>	D	↑	↓	D	↑	↓ <sup>b</sup>	D	↑	↓ <sup>b</sup>
TIMP														
TIMP-1	D	↔	D	↑	↔	D	↑	↔	ND	ND	ND	ND	ND	ND
TIMP-2	D	↔	D	↔	↔	—	—	—	ND	ND	ND	—	—	—

<sup>a</sup>Proteins and/or enzyme activity.

<sup>b</sup>Reduction in the arachidonic acid-treated samples only.

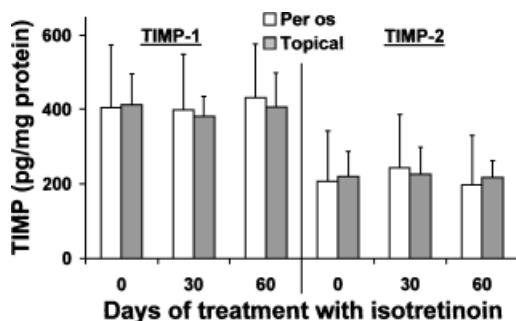
<sup>c</sup>Not statistically significant.

Iso, isotretinoin treated; AA, arachidonic acid treated; D, detected; ND, not detected; ↔, not affected by treatment; ↓, reduced by treatment; ↑, increased by treatment; —, not examined; TIMP, tissue inhibitors of MMP, MMP, matrix metalloproteinases.

genesis (Zouboulis *et al*, 2005). Cells cultured in the presence of arachidonic acid will better represent follicular keratinocytes of acne patients than HaCaT keratinocytes not under inflammatory challenge.

**MMP and TIMP mRNA expression in SZ95 sebocytes** The mRNA expression of MMP and TIMP in SZ95 sebocytes was investigated by analyzing the results of expression profiles generated from untreated SZ95 sebocytes, following complementary DNA (cDNA) microarrays and image analysis (Fig S1). Expression was judged by signal detectability using a negative control sample present on each array. The average proportion of negative samples that was expressed below the probe's signal threshold across the

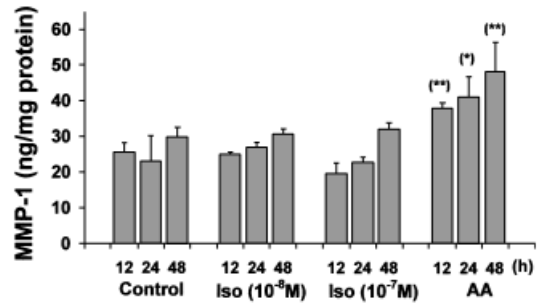
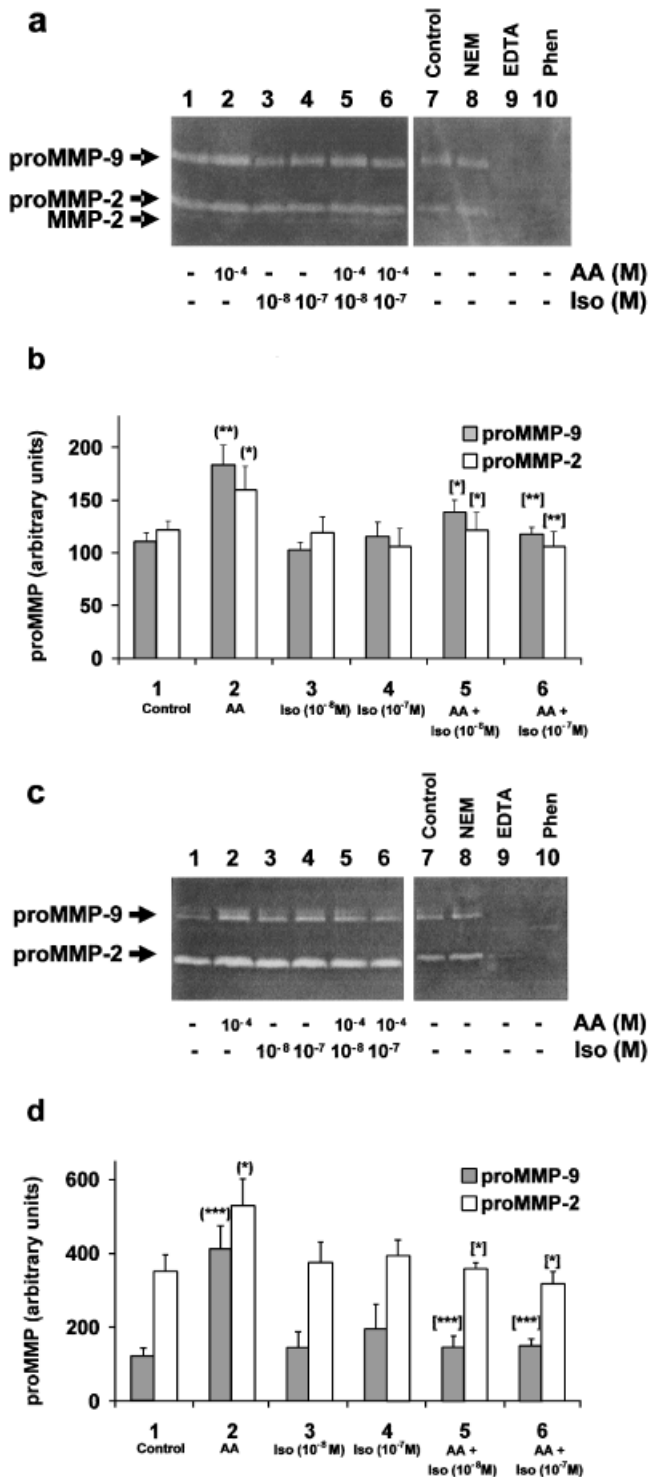
replicate experiments indicated the detectability. The level of 0.9 was used to judge expression of genes. For each cDNA's expression signal, the log ratio (base 2) of this signal was computed with the median expression signal of all 15,657 cDNA on the array. We found that while gelatinases were expressed, mRNA for collagenases MMP-1 and -13 as well as for TIMP-2 was not detected (Table I). Furthermore, SZ95 sebocytes expressed with high probability ( $p > 0.95$ ) mRNA for MMP-7, -14, -15, -21, and -24 (eventually MMP-3 and -11, too) as well as for TIMP-3 and -4, whereas they did not express mRNA for MMP-10, -12, -16, -19, -20, -25, -26, -27, and -28 (Fig S1). Caution should, however, be exercised in interpreting these results, since some of the above-mentioned MMP and TIMP-2 that could not be detected by cDNA microarrays may be expressed if they are examined by more sensitive methods such as quantitative PCR.



**Figure 5**  
**Tissue inhibitor of matrix metalloproteinase (TIMP)-1 and -2 are expressed in sebum samples of facial acne lesions but are not influenced during isotretinoin treatment.** TIMP were measured by ELISA in aliquots of sebum specimens (2  $\mu$ g of protein) during *per os* treatment of 23 acne patients and topical treatment of 36 acne patients with isotretinoin for 0, 30, and 60 d. Each bar represents the mean  $\pm$  SD of triplicate determinations from *n* specimens.

**Gelatinase activity** Gelatin zymography analysis revealed that both HaCaT keratinocytes and SZ95 sebocytes, after 24 h in culture, secreted gelatinase activity, which produced two major lysis bands (Fig 6a and c, respectively, Table I). The upper band comigrated as commercially available proMMP-9, with  $M_r$  corresponding to 87.0 kDa. The lower band comigrated as commercially available proMMP-2, with  $M_r$  corresponding to 68.0 kDa. In HaCaT keratinocytes, the minor lysis band with  $M_r$  65.0 kDa, comigrating as commercially available MMP-2, may represent activation of proMMP-2. In SZ95 sebocytes, minor lysis bands with  $M_r > 87.0$  kDa may be attributed to disulfide polymers of MMP molecules. Gelatinolytic activity was completely inhibited by developing the zymograms in the presence of the metal chelators  $\text{Na}_2\text{EDTA}$  (20 mM) or 1,10-phenanthroline (4 mM), but was unaffected by *N*-ethyl-maleimide (5 mM) (Fig 6a and c), indicating that the activity is due to metal-

loproteinase and excluding serine or cysteine proteinase activity, respectively. These results indicate that the 2 major lysis bands described above are MMP, corresponding to proMMP-9 and -2, respectively (Karakiulakis *et al*, 1988; Visse and Nagase, 2003). Quantification of zymography lysis bands obtained from all cell cultures tested revealed that arachidonic acid induced the secretion of MMP-9 by 45% ( $p < 0.02$ ) and of MMP-2 by 30% ( $p < 0.05$ ) in HaCaT keratinocytes, and by 300% ( $p < 0.01$ ) and 25% ( $p < 0.05$ ), respectively, in SZ95 sebocytes. Zymography analysis of



**Figure 7**

**Matrix metalloproteinase (MMP)-1 is secreted by HaCaT keratinocytes and upregulated by arachidonic acid.** Aliquots of supernatants from HaCaT keratinocytes incubated for 12–48 h were assessed for MMP-1 by ELISA. Each bar represents the mean  $\pm$  SD of triplicate determinations from four assays. Iso, isotretinoin, AA, arachidonic acid ( $10^{-4}$  M). Statistical significance: \* $p < 0.05$ ; \*\* $p < 0.02$ , as compared with control (untreated cells).

samples obtained at 12 or 48 h in culture revealed similar profiles for both cell lines (results not shown), as for 24 h in culture. Isotretinoin (at  $10^{-8}$  and  $10^{-7}$  M) did not affect gelatinolytic activity in keratinocytes or SZ95 sebocytes at any time point (Fig 6, for 24 h in culture). In both cell lines, however, isotretinoin reduced the arachidonic acid-induced secretion of MMP-2 and -9 to levels comparable to controls ( $p < 0.05$  to  $0.01$ ) (Figs 6b and d).

**Secretion of collagenases** Measurement of MMP-1 and -13 by ELISA revealed that HaCaT keratinocytes secreted both MMP-1 ( $29.8 \pm 2.6$  ng per mg protein after 48 h of incubation, Fig 7) and MMP-13 ( $48.1 \pm 2.9$  ng per mg protein after 48 h of incubation; data not shown). In contrast, neither collagenase could be detected in SZ95 sebocytes (Table I). Arachidonic acid significantly increased MMP-1 in HaCaT keratinocytes in a time-dependent manner (Fig 7), but did not influence MMP-13 (data not shown). Isotretinoin (at  $10^{-8}$  and  $10^{-7}$  M) did not influence the basal (Fig 7) or the arachidonic acid-induced MMP-1 secretion in HaCaT keratinocytes up to 48 h of incubation (data not shown). Isotretinoin appeared to reduce MMP-13 production in HaCaT keratinocytes, in a time- and concentration-dependent manner (14%–20% reduction), but this effect was not statistically significant (data not shown).

**Figure 6**

**HaCaT keratinocytes and SZ95 sebocytes secrete promatrix metalloproteinase pro(MMP)-2 and -9, which are upregulated by arachidonic acid and downregulated by isotretinoin.** Representative gelatin zymographies from the cell culture medium of HaCaT keratinocytes (a) and SZ95 sebocytes (c), incubated for 24 h. Lanes 1: untreated (control), lanes 2: arachidonic acid (AA;  $10^{-4}$  M), lanes 3: isotretinoin (Iso;  $10^{-8}$  M), lanes 4: isotretinoin ( $10^{-7}$  M), lanes 5: arachidonic acid ( $10^{-4}$  M) + isotretinoin ( $10^{-8}$  M), lanes 6: AA ( $10^{-4}$  M) + isotretinoin ( $10^{-7}$  M), lanes 7: untreated (control), lanes 8: *N*-ethylmaleimide (NEM, 5 mM), lanes 9:  $\text{Na}_2\text{EDTA}$  (EDTA, 20 mM), and lanes 10: 1,10-phenanthroline (Phen, 4 mM). Arrows indicate proMMP-9 (92 kDa), proMMP-2 (72 kDa), and MMP-2 (64 kDa). Quantitative analysis of the gelatinolytic activity present in the culture media from cell cultures of HaCaT keratinocytes (b) and SZ95 sebocytes (d). Each bar represents the mean  $\pm$  SD from 4 zymograms. Statistical significance: \* $p < 0.05$ ; \*\* $p < 0.02$ , \*\*\* $p < 0.01$ , as compared with control (untreated cells) ( ), or with AA alone [ ]. Iso, isotretinoin; AA, ( $10^{-4}$  M).

**Secretion of TIMP** Measurement of TIMP by ELISA indicated the presence of TIMP-1 ( $16.7 \pm 4.2$  ng per mg protein after 48 h of incubation) and TIMP-2 ( $39.4 \pm 5.5$  ng per mg protein after 48 h of incubation, data not shown) in HaCaT keratinocytes, whereas neither TIMP could be detected in SZ95 sebocytes (Table I, Fig S1). Arachidonic acid ( $10^{-4}$  M), significantly increased TIMP-1 in HaCaT keratinocytes in a time-dependent manner (63% increase after 12 h to 132% increase after 48 h of incubation,  $p < 0.01$ ), but did not influence TIMP-2 (data not shown). Isotretinoin (at  $10^{-8}$  and  $10^{-7}$  M) did not influence either basal TIMP or the arachidonic acid-induced TIMP-1 production in HaCaT keratinocytes up to 48 h of incubation (data not shown).

**Gene expression of MMP and TIMP-1** Gene expression of MMP and TIMP-1 was investigated by RT-PCR analysis. MMP-1, -13, -2, -9, and TIMP-1 mRNA were expressed in HaCaT keratinocytes after 24 h of incubation (Fig 8, Table I), and MMP-2 and -9 in SZ95 sebocytes (Table I). Glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as the internal standard. Quantification of chemiluminescence was performed using a computer-assisted image analysis program. The ratio of chemiluminescence of each parameter measured to GAPDH revealed the following: (a) *HaCaT keratinocytes*: The gene expression of MMP-1 was enhanced by arachidonic acid ( $10^{-4}$  M) by 42%, ( $p < 0.05$ ), but was not affected by isotretinoin ( $10^{-7}$  M). MMP-13 (Fig 8, *inset*) was enhanced by arachidonic acid by 37% and both basal- and arachidonic acid-induced gene expressions were downregulated by isotretinoin by approximately 50% ( $p < 0.05$ ). Arachidonic acid enhanced MMP-2 by 37%

( $p < 0.05$ ), and arachidonic acid-induced gene expression was downregulated by isotretinoin by 33%. MMP-9 was enhanced by arachidonic acid by 36% ( $p < 0.05$ ), and both basal- and arachidonic acid-induced gene expression were downregulated by isotretinoin by 42% and 49%, respectively, ( $p < 0.05$ ). TIMP-1 was enhanced by arachidonic acid by 60% ( $p < 0.05$ ) but was not affected by isotretinoin. (b) *SZ95 sebocytes*: Arachidonic acid enhanced the gene expression of MMP-2 by 38% ( $p < 0.05$ ), and isotretinoin downregulated the arachidonic acid-induced gene expression by 48% ( $p < 0.05$ ). Similarly, arachidonic acid enhanced MMP-9 by 42% ( $p < 0.05$ ), and isotretinoin downregulated the arachidonic acid-induced gene expression by 44% ( $p < 0.05$ ).

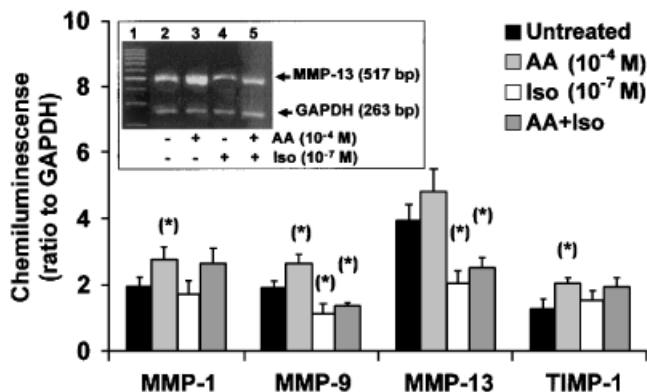
With the exception of MMP-13 (Fig 8, *inset*), however, caution should be exercised in interpreting the changes in gene expression of other MMP and TIMP-1 in treated HaCaT keratinocytes and SZ95 sebocytes, as detected by semi-quantitative RT-PCR, since the magnitude of changes in expression relative to GAPDH were less than 2-fold, which is generally not considered to be significant, despite the statistically significant differences in the relative chemiluminescence values obtained from the ethidium bromide-stained gels.

## Discussion

In this study, we investigated the involvement of MMP and TIMP in sebum from facial acne lesions, and the effect of isotretinoin treatment on these molecules associated with inflammatory matrix responses. Gelatin zymography and western blot analysis indicated the presence of gelatinases in sebum, attributed mainly to proMMP-9, whereas ELISA and western blot analysis revealed the presence of collagenases MMP-1, mainly as proMMP-1 and MMP-13, both in latent and active forms. The expression of TIMP-1 and -2 was also demonstrated using ELISA, and their presence may account for the existence of MMP mainly in the latent form.

The predominant types of cells that could be identified under light microscopy of sebum specimens were keratinocytes, *P. acnes*, and *Staphylococcus epidermidis*. Light microscopy of sebum samples revealed that keratinocytes and bacteria count decreased with progression of treatment with isotretinoin.

The cell source of MMP and TIMP in sebum that we observed cannot be attributed to *P. acnes* or other microorganisms found in the pilosebaceous unit, such as *Pityrosporum ovale*, *Propionibacterium granulosum*, and *S. epidermidis*, since these bacteria do not comprise a source of MMP activity. Therefore, the cell origin of MMP and TIMP in sebum is likely to be keratinocytes. Sebocytes may also be responsible for MMP and TIMP expression in sebum, since the latter is a holocrine product of sebocytes (Zouboulis *et al*, 2003). The possibility that keratinocytes and sebocytes may comprise the cell source for MMP and TIMP in sebum is supported by our findings that HaCaT keratinocytes in culture express and secrete proMMP-2, proMMP-9, MMP-1, MMP-13, and TIMP-1 and TIMP-2, and that SZ95 sebocytes in culture express and secrete pro-



**Figure 8** HaCaT keratinocytes express mRNA for matrix metalloproteinase (MMP) and tissue inhibitor of matrix metalloproteinase (TIMP)-1, and isotretinoin inhibits MMP-9 and MMP-13 *de novo* and arachidonic acid-induced mRNA. Quantitative analysis of chemiluminescence of RT-PCR analyses of HaCaT keratinocytes incubated in the absence or in the presence of isotretinoin ( $10^{-7}$  M, 24 h) using primers for MMP-1, MMP-9, MMP-13, and TIMP-1, as described under "Materials and Methods". Each bar represents the mean  $\pm$  SD from four assays. Statistical significance: \* $p < 0.05$ ; AA, arachidonic acid,  $10^{-4}$  M; Iso, isotretinoin,  $10^{-7}$  M. Inset: Representative analysis of chemiluminescence of RT-PCR analyses of HaCaT keratinocytes incubated in the absence or in the presence of isotretinoin ( $10^{-7}$  M, 24 h) using primers for MMP-13. GAPDH of expected size 263 bp was used as an internal standard. The expected size of the reaction product for MMP-13 is indicated by the arrow. Lane 1, DNA ladder (100 bp); lane 2, untreated; lane 3, AA ( $10^{-4}$  M); lane 4, isotretinoin ( $10^{-7}$  M); and lane 5, AA ( $10^{-4}$  M) and isotretinoin ( $10^{-7}$  M).

MMP-2 and -9, which was also confirmed by cDNA microarray analysis. Sebocytes may be a source of other MMP, which may also contribute to the pathogenesis of acne. These findings are in agreement with reports that human keratinocytes of normal or pathological origin produce or express latent and active gelatinases and TIMP (Baumann *et al*, 2000; Fleischmajer *et al*, 2000; Kobayashi *et al*, 2000).

ProMMP-2 was not detected in sebum, even though both HaCaT keratinocytes and SZ95 sebocytes produced proMMP-2. This may be attributed to the fact that cells in culture are not confronted with proteases at the level that inflammatory human tissue does. In the latter case, the presence of proteases may lead to deactivation, inhibition, or destruction of MMP-2 in the diseased human follicle *in vivo*. Even though the results from HaCaT keratinocytes and SZ95 sebocytes in culture are of considerable value, caution should be exercised in extrapolating the results from cell cultures to the *in vivo* situation, since HaCaT cells are not a model for follicular keratinocytes and differences may exist in MMP expression between normal and HaCaT keratinocytes. Thus, the possibility that the cell sources of sebum MMP and TIMP are follicular keratinocytes remains to be verified by using tissues of sebaceous follicle and glands of acne patients.

The prospect that other type of cells associated with MMP and TIMP production, such as skin fibroblasts or infiltrating macrophages in the inflammatory acne lesions, may comprise the cell source of MMP and TIMP in sebum should also be considered. In this respect, it has been reported that retinoids reduce the activation of MMP in fibroblasts (Overall, 1995) and that tretinoin downregulates MMP-9 mRNA and protein in alveolar macrophages from patients with chronic obstructive pulmonary disease (Frankenberger *et al*, 2001) and emphysema (Mao *et al*, 2003), and that it reverses upregulation of MMP-13 in human keloid-derived fibroblasts (Uchida *et al*, 2003).

The pro-inflammatory essential fatty acid arachidonic acid significantly induced proMMP-2 and -9 in both cell types investigated, as well as MMP-1 and TIMP-1 in HaCaT keratinocytes. The association between arachidonic acid and MMP and TIMP expression has also been reported for several cell types and tissues, as e.g., in the human atherosclerotic plaques (Cipollone *et al*, 2003), in synoviocytes (Burger *et al*, 2003), in renal tubular cells (Cussac *et al*, 2002), and in calvaria and bone marrow cells (Choi *et al*, 2003).

Topical or systemic treatment of acne patients with isotretinoin did not affect MMP-1 or TIMP but resulted in reduced activity of MMP-9 and secretion of MMP-13 in sebum, an effect that was enhanced with the duration of treatment and in parallel to the improvement of the clinical picture. The effects of *per os* or topical administration of isotretinoin on proMMP-9 of sebum samples that we observed are in good agreement with reports that *per os* administration of all-*trans*-RA to patients with emphysema reduced plasma MMP-9 protein and activity, while having little effect on TIMP-1 levels (Mao *et al*, 2003), and that topical administration of retinol in healthy humans reduced MMP-9 activity in punch biopsies of human buttock skin (Varani *et al*, 2000).

The effects of isotretinoin in sebum MMP and TIMP following topical or systemic treatment of acne patients correlate with the effects induced by isotretinoin in arachidonic acid-treated HaCaT keratinocytes, which may better represent follicular keratinocytes of acne patients than HaCaT keratinocytes not under inflammatory challenge. Isotretinoin significantly inhibited the arachidonic acid-induced proMMP-2 and -9 and MMP-13 secretion by HaCaT keratinocytes, although the latter was not statistically significant, as well as the arachidonic acid-induced mRNA of MMP-9 and -13. In SZ95 sebocytes, isotretinoin also inhibited the arachidonic acid-induced increase of proMMP-2 and -9 secretion, and the arachidonic acid-induced expression of mRNA for MMP-9. The above-described effect of isotretinoin on MMP production by keratinocytes and sebocytes is in good agreement with studies on the effect of RA on various cell cultures or tissues, which demonstrated: (a) that all-*trans*-RA inhibited the activity of MMP-2 and -9 in human arterial smooth muscle cells (Axel *et al*, 2001), the activity of MMP-9 in human bronchoalveolar lavage cells (Frankenberger *et al*, 2001), the activity of MMP-2 and -9 in a rabbit model of vein bypass grafting (Leville *et al*, 2000), and pro- and active MMP-9 protein in myelogenous leukemia cell lines (Devy *et al*, 2002); (b) that retinol and all-*trans*-RA decreased 98–96, 72–68, and 46–45 kDa gelatinolytic activity in capillary endothelial cells (Braunhut and Moses, 1994); (c) that retinoids reduced the activation of MMP in fibroblasts (Overall, 1995), and (d) that locally produced all-*trans*-RA suppresses MMP during endometrial differentiation (Osteen *et al*, 2002). Similarly, the lack of any effect of isotretinoin on TIMP secretion in sebum or in cell cultures is in agreement with reports indicating that oral all-*trans*-RA did not affect TIMP-1 in a rabbit model of vein bypass grafting (Leville *et al*, 2000), and that it did not influence expression of TIMP-1 in human primary melanoma (Jacob *et al*, 1998).

The inhibition of proMMP-9 following treatment with isotretinoin is not due to a direct inhibitory effect of the drug on the enzyme, since isotretinoin, up to 1 mM, did not exhibit any direct effect on proMMP-9 isolated from sebum specimens of facial acne lesions or on commercially available gelatinases. The decrease in proMMP-9 and MMP-13 that we observed following treatment with isotretinoin may be attributed to the reduction of the expression rate of these MMP. This is in agreement with our observation that isotretinoin downregulated MMP-9 and -13 mRNA in HaCaT keratinocytes and MMP-9 in SZ95 sebocytes. It is also supported by reports that RA reduces the mRNA expression of MMP-2 and/or -9 in human arterial (Axel *et al*, 2001) and rat aortic (Neuville *et al*, 1999) smooth muscle cells, in tumor (Tsang and Crowe, 2001) and leukemia (Devy *et al*, 2002) cell lines, and in a rabbit model of vein bypass grafting (Leville *et al*, 2000), whereas tretinoin reverses upregulation of MMP-13 in human keloid-derived fibroblasts (Uchida *et al*, 2003).

The presence of MMP-9 activity and protein, and of collagenases MMP-1 and -13 and TIMP protein of epithelial origin in acne sebum, and the isotretinoin-induced decrease of proMMP-9 and MMP-13 in parallel to the clinical improvement of the lesions indicate that these MMP and TIMP may be involved in the pathophysiology of acne lesions,



possibly by contributing to abnormal hyperproliferation, and degradation and remodeling of extracellular matrix structures, such as the basement membrane of the acroinfundibulum of the sebaceous follicle and the sebaceous glands. Although the precise functional role of MMP and TIMP in acne pathology remains to be clarified, it appears that the isotretinoin-induced reduction in proMMP-9 and MMP-13, via mechanisms that do not affect TIMP, may contribute to the therapeutic effects of this agent in acne.

## Materials and Methods

**Patients** Lesions in acne vulgaris patients ranged from clinically non-inflammatory microcomedones, closed or open comedones, to inflammatory papules, pustules, and cysts, intermingled to various extents. The criteria of the Global Alliance to Improve Outcomes in Acne were used to classify acne into mild, moderate, and severe (Gollnick *et al*, 2003). Fifty-nine female patients with acne vulgaris, aged  $17.8 \pm 1.7$  y (mean  $\pm$  SD) were treated with topical (0.05% once daily, 36 patients with mild and moderate papulopustular acne) or systemic (1 mg per kg per d *per os*, 23 patients with moderate nodular or severe acne) administration of isotretinoin for 3–4 mo, after providing their written consent. The medical history of all patients was free from recent microbial infections or any other disorders of the skin. Patients had not been treated with retinoids in the past and were not under any other medication for at least 3 mo prior to the initiation of the treatment in this study. All patients were thoroughly informed about the adverse effects of isotretinoin treatment and received other, but no hormonal, contraception during and 3 mo after discontinuation of the treatment. Participants gave their written informed consent. The medical ethical committee of Aristotle University of Thessaloniki has approved all the described studies. The study was conducted according to the Declaration of Helsinki Principles.

**Sebum samples** Sebum from facial lesions, such as comedones, papules, and pustules, were collected under standardized conditions from individual acne lesions. Sebum collection was performed using sterile, blunt, plastic "spatulas" to rupture the lesion and gently squeeze out the sebum. Sebum was collected at the tip of the spatula and transferred into sterile 1 mL Eppendorf plastic vials. Samples were pooled in one plastic vial per individual patient, per time point, and stored at  $-70^{\circ}\text{C}$  until use. Sebum sampling was performed at three time points: prior to treatment (0 d sample),  $31 \pm 3$  d (30 d sample), and  $63 \pm 4$  d (60 d sample) after treatment with isotretinoin. Aliquots of sebum samples, diluted to contain the same amount of protein per mL, were analyzed in a blinded fashion for the presence or absence of keratinocytes and/or bacteria. Light microscopy of sebum samples was performed using an Olympus BX50 microscope (Tokyo, Japan). Bacterial cultures of sebum specimens obtained before and during treatment were performed under strict aseptic techniques in Columbia blood agar and anaerobe blood agar. Bacteria were identified on the basis of morphological and biochemical characteristics employing the VITEK system (bioMérieux, Marcy l'Etoile, France). Identification was confirmed by testing for various properties using tests according to Murray *et al* (1999).

**Cultures and treatment of HaCaT keratinocytes and SZ95 sebocytes** Spontaneously immortalized, nontumorigenic human HaCaT keratinocytes (Boukamp *et al*, 1988) (generously provided by Prof. N. E. Fusenig and Dr D. Breitkreuz, German Cancer Research Center, Heidelberg, Germany) and immortalized human facial SZ95 sebocytes (Zouboulis *et al*, 1999) were seeded at a concentration of  $3 \times 10^4$  cells per well in 24-well culture plates (Nunc, Wiesbaden, Germany) and were maintained in Sebomed medium (Biochrom, Berlin, Germany) containing 10% (vol/vol) fetal calf serum, 5 ng per mL recombinant human epithelial growth factor, and

penicillin/streptomycin (all from Biochrom) at 5%  $\text{CO}_2$  and  $37^{\circ}\text{C}$ . Cells were cultured for 2 d in the presence or absence of arachidonic acid (Sigma-Aldrich, Deisenhofen, Germany), and dissolved in ethanol to give a final arachidonic acid concentration of  $10^{-4}$  M. The final concentration of ethanol in medium without and with arachidonic acid was 0.1%. Subsequently, cells were treated with isotretinoin (Sigma-Aldrich) dissolved in dimethyl sulfoxide to give final isotretinoin concentrations of  $10^{-8}$  and  $10^{-7}$  M. The final concentration of dimethyl sulfoxide in medium without and with isotretinoin was 0.2%. Isotretinoin was handled under dimmed yellow light. Culture supernatants were collected after 12, 24, and 48 h of treatment with isotretinoin or DMSO into sealed plastic tubes and frozen at  $-40^{\circ}\text{C}$  until further evaluation. Cells were also collected separately after 48 h of treatment and stored under similar conditions.

**Gelatin zymography** Gelatin zymography analysis was performed in sebum specimens and medium of cell cultures, collected as described above. In addition, gelatin zymography was performed following *in vitro* experiments to test the effects of the agent on gelatinases present in sebum as well as on commercially available gelatinases. Sebum samples were suspended in 1 mL ddH<sub>2</sub>O and subjected to ultrasonication in a Clifton Ultrasonic bath (Nickel-Electro, Weston-Super-Mare, North Somerset, UK) ( $3 \times 5$  min) and precipitation from saturation with 25%–50%  $(\text{NH}_4)_2\text{SO}_4$  at  $4^{\circ}\text{C}$  (Karakiulakis *et al*, 1988). For cell cultures, aliquots of the supernatants were diluted to contain the same amount of protein per mL. The gelatinolytic activity of MMP was determined by gelatin zymography analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing but non-reducing conditions (Karakiulakis *et al*, 1997). Molecular sizes of bands displaying enzymatic activity were estimated in comparison with purified proMMP-2 (72.0 kDa), active MMP-2 (64.0 kDa), proMMP-9 (92.0 kDa) and active MMP-9 (68.0 kDa) (Anawa Trading, Wangen). The pre-stained standard protein molecular weight markers used were: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), L-glutamic dehydrogenase (55.0 kDa), ovalbumin (42.7 kDa) and aldolase (40.0 kDa) (all from Promega, Madison, WI). Gelatinolytic activity was quantified using a computer-assisted image analysis program (1D Image Analysis Software, version 3.0 of Kodak Digital Science, Eastman Kodak, Rochester, New York). The nature of the proteolytic bands was further characterized by including specific protease inhibitors:  $\text{Na}_2\text{EDTA}$  (20 mM), 1,10-phenanthroline (4 mM), or *N*-ethyl-maleimide (5 mM) (all obtained from Sigma-Aldrich) in the enzyme incubation buffer. The effect of isotretinoin on 0 d sebum samples (corresponding to 5  $\mu\text{g}$  protein) or purified latent MMP-2 and -9 (1 ng protein) was studied by pre-incubating samples with isotretinoin (0–1 mM) for 30 min at room temperature, followed by gelatin zymography. SDS was then removed from the gels by equilibrating ( $2 \times 30$  min) in 2.5% (vol/vol) Triton X-100 (Sigma-Aldrich) and gels were incubated in enzyme buffer (50 mM Tris-HCl, pH 7.3, containing 200 mM NaCl, 5 mM  $\text{CaCl}_2$  and 0.1% Triton X-100), in the presence of isotretinoin (0–1 mM) for 18 h, at  $37^{\circ}\text{C}$ .

**Western blot analysis for determination of MMP** Sebum samples, containing the same amount of total protein, were enriched in MMP by precipitation with  $(\text{NH}_4)_2\text{SO}_4$  (60% saturation) (Karakiulakis *et al*, 1988); the resultant precipitates were dissolved in Laemmli sample buffer containing 5%  $\beta$ -mercaptoethanol, boiled for 5 min, and then subjected to SDS-PAGE (Laemmli, 1970) on 10% polyacrylamide gels. After electrophoresis, the separated proteins were electro-transferred onto nitrocellulose membranes according to the method of Towbin *et al* (1979). The free binding sites on the nitrocellulose membranes were blocked with 5% skim milk in 20 mM Tris-HCl, pH 7.4/150 mM NaCl buffer (TBS), containing 0.05% Tween-20 (TBS-T), at room temperature for 1 h. After three 10 min washes with TBS-T, the membranes were incubated with rabbit antiserum produced against human MMP-2 or MMP-9, (a generous gift from Dr. P. Koolwijk, Gaubius Lab. TNO-PG, the Nether-

lands) (Hanemaaijer *et al*, 1998), or affinity purified anti-rabbit polyclonal AB806 for MMP-1 and AB8114 for MMP-13 (Chemicon International, Temecula, California), at dilution 1:1000, in TBS-T, containing 1% skim milk, at 4°C for 20 h. After washing three times with TBS-T, they were incubated with peroxidase-conjugated goat anti-rabbit IgG at dilution 1:4000 in TBS-T, containing 1% skim milk, at room temperature for 2 h. Then, the membranes were washed with TBS-T three times, once with TBS and the immunoreacted proteins were detected by the enhanced chemiluminescence method, according to the manufacturer's instructions (Pierce, Rockford, Illinois). For negative controls, nitrocellulose membranes were subsequently treated with stripping solution (Chemicon). After stripping, membranes were re-blocked, as described above, and incubated with rabbit anti-ovalbumin IgG (Chemicon) (instead of the specific rabbit antibodies for MMP), at dilution 1:1000, in TBS-T, containing 1% skim milk, at 4°C for 20 h. Subsequent steps were as described above. When the non-specific antibody was used, no immunoreactive bands could be detected for any MMP examined. Quantification of chemiluminescence was performed using a computer-assisted image analysis program (1D Image Analysis Software, version 3.0 of Kodak Digital Science). Molecular size was estimated by comparison prestained standard proteins: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa) and soybean trypsin inhibitor (21.5 kDa) (all from Promega, Madison, Wisconsin), which were electrophoresed under reducing conditions.

**ELISA for determination of collagenases and TIMP** MMP-1 and -13 were determined in aliquots of sebum samples or the supernatants of cell cultures by ELISA. Samples were enriched in MMP by precipitation with  $(\text{NH}_4)_2\text{SO}_4$  (60% saturation) (Karakioulakis *et al*, 1988) and assayed for MMP-1 and -13 as previously described (Papakonstantinou *et al*, 2003), using anti-MMP-1 (1  $\mu\text{g}$  per mL, Ab 806, Chemicon) or anti-MMP-13 (1  $\mu\text{g}$  per mL, Ab 8114, Chemicon) polyclonal antibodies in PBS-T and peroxidase-conjugated second antibody (goat anti-rabbit IgG, Chemicon). The concentration of collagenases was estimated in pg collagenase protein per  $\mu\text{g}$  of total protein per sebum sample, using reference samples of MMP-1 and -13 from human periprosthetic tissue of loose hip endoprostheses (Syggelos *et al*, 2001). TIMP-1 and -2 were measured in aliquots of sebum samples or the supernatants of cell cultures, using ELISA systems (Biotrak RPN 2611 for TIMP-1 and Biotrak RPN 2618 for TIMP-2; Amersham Pharmacia, Freiburg, Germany) that recognize total human TIMP-1 or -2, both free and that complexed with MMP.

**RT-PCR for expression of MMP and TIMP-1** Isolation of RNA was performed by the RNeasy spin mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Gene expression of MMP and TIMP-1 was ascertained by semi-quantitative RT-PCR analysis according to Papakonstantinou *et al* (2004), adjusted as follows: total RNA (50 ng for MMP-1, 400 ng for MMP-13, 200 ng for MMP-2, 200 ng for MMP-9, 20 ng for TIMP-1, and 10 ng for GAPDH) was added to each RT reaction containing one-step RT-PCR mix (Robus I, Finnzymes, Espoo, Finland) and the appropriate PCR primers (15 pmole) in 50  $\mu\text{L}$  total volume. Reverse transcription was carried out for 30 min at 50°C, followed by a 2 min step at 94°C. PCR was then performed on a PTC-100 programmable Thermal Controller (MJ Research, Waltham, Massachusetts), programmed for several cycles of 1 min at 94°C, 1 min at optimal annealing temperature, and 1 min at 72°C, followed by a 10 min step of extra extension at 72°C. The amount of total RNA used, as well as the number of PCR cycles, was adjusted so that each PCR amplification was in the linear range. Primers for MMP-1 (428 bp, annealing at 55°C, 30 cycles, Kontinen *et al*, 1999), MMP-2 (605 bp, annealing at 58°C, 40 cycles, Giamberti *et al*, 1998), MMP-9 (519 bp, annealing at 55°C, 30 cycles, Moore *et al*, 2000), MMP-13 (517 bp, annealing at 53°C, 35 cycles, Moore *et al*, 2000), and TIMP-1 (534 bp, annealing at 60°C, 30 cycles, Moore *et al*, 2000) were obtained from MWG-Biotech AG (Ebersberg, Germany). Am-

plification specific for GAPDH (MWG-Biotech AG) (263 bp, annealing at 61°C, 30 cycles) was used to estimate the efficiency of the reverse transcription reaction for MMP and TIMP-1. Five microliters of each PCR reaction mixture were analyzed in a 2% agarose gel, using a 100 bp DNA ladder (Invitrogen, Life Technologies, Carlsbad, California). Visualization of DNA bands was achieved with UV illumination of ethidium bromide-stained gels. Quantification of chemiluminescence was performed using a computer-assisted image analysis program (1D Image Analysis Software, version 3.0 of Kodak Digital Science Eastman Kodak).

**Analysis of MMP and TIMP expression by cDNA microarrays** SZ95 sebocytes were maintained under the conditions described above without arachidonic acid and isotretinoin for 120 h. Subsequently, RNA extraction was performed using the RNeasy Midi kit (Qiagen) according to the manufacturer's protocol. RNA was photometrically measured in a Pharmacia GeneQuant II spectrophotometer (Freiburg, Germany) and stored at -80°C until use. Amplified RNA (aRNA) was generated from 3  $\mu\text{g}$  of DNase 1-treated total RNA using the MegaScript T7 High Yield Transcription kit (Ambion, Austin, Texas). RNA purity, integrity and concentrations were evaluated on the Agilent 2100 bioanalyzer. Cy3- and Cy5-labelled cDNA was reverse transcribed from 3  $\mu\text{g}$  of aRNA per reaction. All labeling reactions used the Cyscribe First-Strand cDNA labeling Kit (Amersham Pharmacia). Purification of labeled cDNA was carried out using Microcon YM-30 columns (Millipore, Billerica, Massachusetts). Four replicated hybridizations consisting of duplicated dye swaps were carried out on the Human ENSEMBL chip using protocols described previously (Adjaye *et al*, 2004). The cDNA microarray consists of 15,500 non-redundant, fully sequenced, annotated human cDNA (Human Ensembl set RZPD1.1) spotted in duplicate on superAmine-coated glass slides (Telechem, Sunnyvale, California) with each slide containing positive/internal controls ( $\beta$ -actin, HPRT), and a selection of Arabidopsis cDNA as negative controls. Slides were scanned using the Affymetrix 428 Array Scanner (Santa Clara, California).

**Data analysis of cDNA microarrays** Image analysis was carried out with the AIDA Array Matrix software (Raytest, Straubenhardt, Germany). In total, 11 replicate experiments were carried out. Data were normalized as described in Herwig *et al* (2001). In order to judge whether a given gene was expressed in SZ95 sebocytes, for each experiment, the signal of the gene was compared with a reference distribution derived from 3626 negative control signals by computing the proportion of negative spots having a smaller signal than the gene of interest. The average proportion across all replicates was defined as the "expression probability" for the gene and was used as an indicator for the expression strength in SZ95 sebocytes. Visual inspection of hybridization images indicates that a high probability ( $p > 0.95$ ) corresponds to visible signals, whereas spots with  $p < 0.9$  correspond to absent signals. Probabilities in between correspond to weakly expressed signals.

**Protein measurement** Protein content was determined in aliquots of sebum specimens, aliquots of cell culture supernatants and cells with the standard Bradford assay (Bio-Rad, Glattbrugg, Switzerland) using bovine serum albumin (Sigma-Aldrich Chemie, Steinheim, Germany) as standard. All data presented were normalized per protein content for each sebum or cell culture specimen.

**Statistical analysis** Where relevant, data are presented as mean  $\pm$  SD. Differences between means were evaluated by analysis of variance.  $p < 0.05$  was considered statistically significant.

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### Supplementary Material

The following material is available online for this article.

**Figure S1** Expression of MMPs and TIMPs in SZ95 sebocytes measured with DNA microarrays

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