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Human Keratinocyte Cell Lines Differ in the Expression of the Collagenolytic Matrix Metalloproteinases-1, -8, and -13 and of TIMP-1

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We investigated cells and conditioned media of the three human keratinocyte cell lines HaCaT (non-tumorigenic), A5 (benign, tumorigenic) and II-4RT (malignant, tumorigenic) with regard to production and secretion of the collagenases-1 to -3 (MMP-1, MMP-8 and MMP-13) and TIMP-1 using semi-nested RT-PCR, Western blots, ELISA, immunocytochemistry and casein zymography.

Transcripts of MMP-1, -8, -13 and TIMP-1 were detected in all cell lines by RT-PCR and the corresponding proteins were found in the cytoplasm of all three cell lines by Western blot analysis and/or immunocytochemistry. The conditioned media of the malignant II-4RT cells contain significantly more MMP-1 and MMP-8 than those of HaCaT or A5 as evidenced by immunoblotting and ELISA. In addition to the presence of latent MMP-1, zymography also detected the active form of this enzyme. TIMP-1 was found only in extracts of all three cell lines, predominantly in A5.

This study clearly indicates that the epithelial tumor cells synthesize different collagenases and TIMP-1. The malignant clone secretes increased amounts of distinct collagenases compared to the non-tumorigenic cell line, thereby verifying a correlation between biological behaviour and the amount of collagenases. In addition, we provide clear evidence that MMP-8 is not exclusively found in polymorphonuclear granulocytes, but also in keratinocyte cell lines.

Key words: Collagenase / HaCaT cells / *In vitro* study / Keratinocyte cell lines / MMP / Tumorigenicity.

Introduction

The process of tumor invasion requires degradation of the extracellular matrix by proteolytic enzymes (Matrisian 1992; Birkedal-Hansen *et al.*, 1993). Therefore, the balance between production, activation and inhibition of these enzymes is fundamental for the steps involved in the tumor-associated matrix turnover (Schmitt *et al.*, 1992; Mignatti and Rifkin, 1993; Johnsen *et al.*, 1998). In particular, an increased expression of matrix metalloproteinases (MMPs) has been shown to be involved in tumor invasion and metastasis (Basset *et al.*, 1997; Johnsen *et al.*, 1998).

Until now more than 17 members of the family of zinc-dependent MMPs have been identified (Woessner, 1998). Due to their substrate specifity and structure, MMPs can be classified into collagenases, stromelysins, gelatinases, membrane-type MMPs and other MMPs (Kähäri and Saarialho-Kere, 1997). During tumor invasion, collagenases are particulary important as these enzymes are able to degrade the collagenous connective tissue which represents the bulk of the interstitial matrix.

The three mammalian collagenases known, *i. e.* the fibroblast-type interstitial collagenase, the neutrophil collagenase, and collagenase-3 differ in their substrate preferences. The fibroblast-type interstitial collagenase (MMP-1, collagenase-1; Goldberg *et al.*, 1986) prefers type III collagen over type I, II, VII and X (Gadher *et al.*, 1989; Seltzer *et al.*, 1989). It is widely expressed in various cell types including fibroblasts, chondrocytes, macrophages, endothelial cells and keratinocytes (Freije *et al.*, 1994). Increased levels of this enzyme have been detected in various carcinomas, *e. g.* thyroid and gastric cancer (Kameyama, 1996, Sakurai *et al.*, 1997a, b).

The neutrophil collagenase (MMP-8, collagenase-2; Hasty *et al.*, 1987) preferentially cleaves type I and II collagens, but also type III collagen. This enzyme was thought to be synthesized exclusively by polymorphonuclear neutrophils (PMN) where it is stored as a latent proenzyme in the specific granules (Murphy *et al.*, 1977; Mainardi *et al.*, 1991). However, *in vivo* MMP-8 expression has recently been observed in mononuclear fibroblast-like cells in the rheumatoid synovial membrane, and MMP-8 mRNA was detected in cultured rheumatoid synovial fibroblasts, hu-

man endothelial cells and human chondrocytes (Chubinskaya *et al.*, 1996; Cole *et al.*, 1996; Hanemaaijer *et al.*, 1997).

The collagenase-3 (MMP-13; Freije *et al.*, 1994) shows an exceptionally wide substrate specifity compared to other MMPs. In addition to fibrillar type I, II and III collagens it also degrades type IV, IX, X and XIV collagen, gelatin, tenascin, fibronectin and proteoglycan coreproteins (Knäuper *et al.*,1996; Mitchell *et al.*,1996). Collagenase-3 is expressed by different malignant tumors including breast carcinoma (Freije *et al.*, 1994) and squamous cell carcinoma of the skin, head and neck (Airola *et al.*, 1997; Johansson *et al.*, 1997a; Cazorla *et al.*, 1998).

Activation of MMPs is controlled by specific inhibitors, termed 'tissue inhibitors of metalloproteinases' (TIMPs). So far 4 TIMPs have been characterized which differ in their inhibitory activities for the various MMPs. Thus, TIMP-1 predominantly inhibits MMP-1, -3 and -9 (Mackay *et al.*, 1992), while TIMP-2 preferentially binds to MMP-2 (Goldberg *et al.*, 1989). Besides these inhibitory effects, TIMPs exert other biologically relevant functions and even appear to be involved in the activation of certain MMPs.

So far it is unclear to what extent epithelial tumor cells and the neighbouring stromal cells are responsible for the elevated levels of collagenases in cancer tissues. In order to elucidate the contribution of the tumor cells, we investigated whether the differences in tumorigenicity in a model system of three closely related human keratinocyte cell lines (Boukamp *et al.*, 1988, 1990) are reflected by their profiles of constitutively expressed collagenases.

The cell lines investigated were HaCaT, a spontaneously immortalized human keratinocyte cell line (Boukamp *et al.*, 1988), and the two clones A5 and II-4RT which were derived from HaCaT by stable transfection with the cellular Ha-*ras* oncogene (Boukamp *et al.*, 1990). When implanted into athymic mice HaCaT cells are non-tumorigenic, A5 cells form benign cysts and II-4RT cells develop locally invasive squamous cell carcinomas. Thus the three cell lines represent a unique model of closely related cells with defined *in vivo* tumorigenicity.

Our approach is based on recent observations that in this model the amounts of gelatinases (MMP-2, -9) and stromelysins (MMP-3, -7, -10) synthesized and secreted increase with tumorigenicity, thus providing a potential link between these enzymes and the cellular behaviour (Bachmeier *et al.*, 1998; 2000).

In the present study we provide evidence that all cell lines express the collagenases MMP-1, -8 and -13 as well as TIMP-1. Thus, MMP-8, the neutrophil collagenase, is not specific for leukocytes, but can also be expressed by epithelial tumor cells. The locally invasive cell line secretes increased amounts of MMP-1 and -8; furthermore, in addition to the latent enzyme also active MMP-1 is present in the media. These observations suggest that the collagenases expressed by epithelial tumor cells may directly contribute to invasive tumor growth. In addition, the presence of several collagenases indicates the redundancy of this proteolytic system.

Results

Detection of mRNAs of Collagenases and TIMP-1

Analysis of total RNA by semi-nested RT-PCR demonstrated that the three cell lines express MMP-1 (Figure 1, lanes 1 and 2), MMP-8 (lanes 3 and 4), MMP-13 (lanes 5 and 6) and TIMP-1 (lanes 7 and 8). Primers were chosen so that the PCR amplicons included exon-exon transitions (see Table 1) and thus amplification of genomic DNA would result in products easily recognizable by their size. In addition, the identity of the amplicons was verified by digestion with restriction endonucleases (Table 1, Figure 1).

Detection of Collagenases and TIMP-1 in Cells and Cell Extracts

Protein expression of MMP-1, -8, -13 and TIMP-1 was detected in extracts and conditioned media (see section below) of all three cell lines by means of Western blots, immunohistochemistry, ELISA, and zymography; the high

Table 1 Primers Used for PCR Amplification.

Primer	Sequence	Location
MMP-1 forward	5′-AGATGTGGAGTGCCTGATGT-3′	exon 2
MMP-1 reverse (outer)	5′-CCTGCAGTTGAACCAGCTAT-3′	exon 9
MMP-1 reverse (inner)	5′-GTGCGCATGTAGAATCTGTC-3′	exon 7
MMP-8 forward	5′-ACCAATTACCAAGCAACCAG-3′	exon 2
MMP-8 reverse (outer)	5′-GGGATACATCAAGGCACCAG-3′	exon 5
MMP-8 reverse (inner)	5′-GAGCAGCAACAAGAAACAAG-3′	exon 5
MMP-13 forward	5′-CCAACCCTAAACATCCAAAAAC-3′	exon 6
MMP-13 reverse (outer)	5′-CACCACAAAATGGAATTTGCTG-3′	exon 9
MMP-13 reverse (inner)	5′-GGCATGACGCGAACAATAC-3′	exon 9
TIMP-1 forward	5′-CCAGAGAGACACCAGAGAAC-3′	exon 1/2
TIMP-1 reverse (outer)	5′-GAGGTAAGTGCCATGGTGAG-3′	exon 2
TIMP-1 reverse (inner)	5′-ACTCACCGAGGTCGGAATTG-3′	exon 2

All primers were designed using the GenBank cDNA sequences for MMP-1 (accession number U78045), MMP-8 (accession number J05556), MMP-13 (accession number X75308), and TIMP-1 (accession number Y09720).

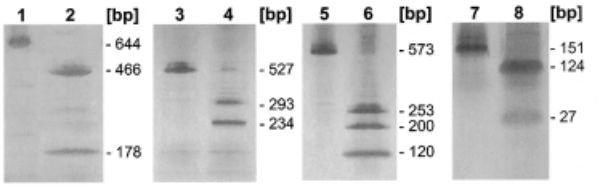

Fig. 1 Detection of MMP-1, -8, -13, and TIMP-1 mRNA by Semi-Nested RT-PCR.
Data are shown for MMP-1 (lanes 1 and 2), MMP-8 (lanes 3 and 4), MMP-13 (lanes 5 and 6) and TIMP-1 (lanes 7 and 8). Reverse transcription and semi-nested PCR were performed with total RNA from about 10^6 cells. Aliquots of the PCR mixtures before (lanes 1, 3, 5 and 7) and after digestion with restriction endonucleases (lanes 2, 4, 6 and 8) were subjected to PAGE. The endonucleases *Sty*I, *Fok*I, *Dde*I and *Hae*III were used to digest amplicons from MMP-1, -8, -13, and TIMP-1, respectively. The gels shown are the results from HaCaT cells, representatively for all three cell lines.

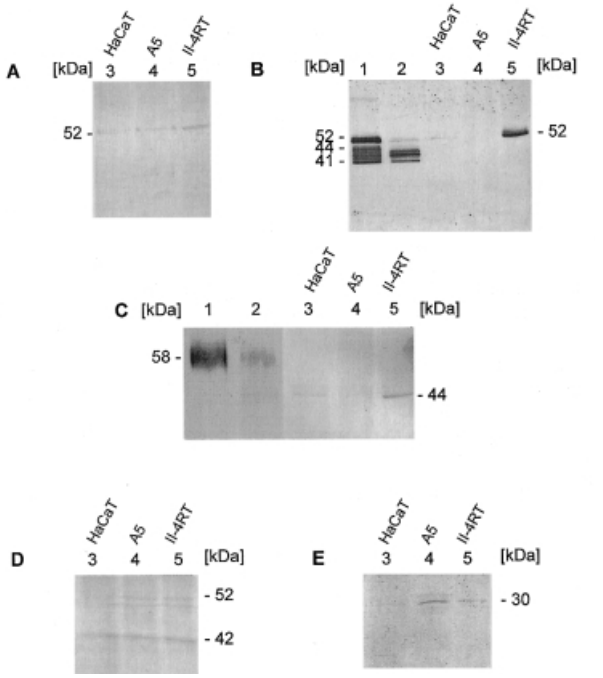

Fig. 2 Western Blot Analyses of Cell Extracts and Serum-Free Conditioned Media of HaCaT, A5 and II-4RT Cells Using Antibodies Specific for MMP-1, -8, -13, and TIMP-1.
Extracts (60 µg protein) analyzed for MMP-1 (A), MMP-13 (D), and TIMP-1 (E). Conditioned Media (15 µg protein) analyzed for MMP-1 (B), and MMP-8 (C). HaCaT cells: lanes 3; A5 cells: lanes 4; II-4RT cells: lanes 5; MMP-1 and -8 controls (B, C): lanes 1: without APMA; lanes 2: after APMA treatment.

sensitivity of zymography and immunohistochemistry allowed to detect distinct proteins where the limits of the Western blot technique were already reached.

Western Blots MMP-1 was detected as faint bands of latent enzyme (52 kDa, Figure 2A) and MMP-13 as bands of latent (52 kDa, Figure 2D) as well as active enzyme (42 kDa, Figure 2D). About equal amounts were found in extracts of all three cell lines. MMP-8 was not detected in the extracts of any cell line by immunoblotting.

In addition, bands of TIMP-1 at ~ 30 kDa were detectable in extracts of all three cell lines. The intensity of the TIMP-1 band was highest in benign A5 cell extracts (Figure 2E; lane 3), lower in those of malignant II-4RT (Figure 2E; lane 4) and hardly detectable in those of HaCaT (Figure 2E; lane 1) (see also Table 2).

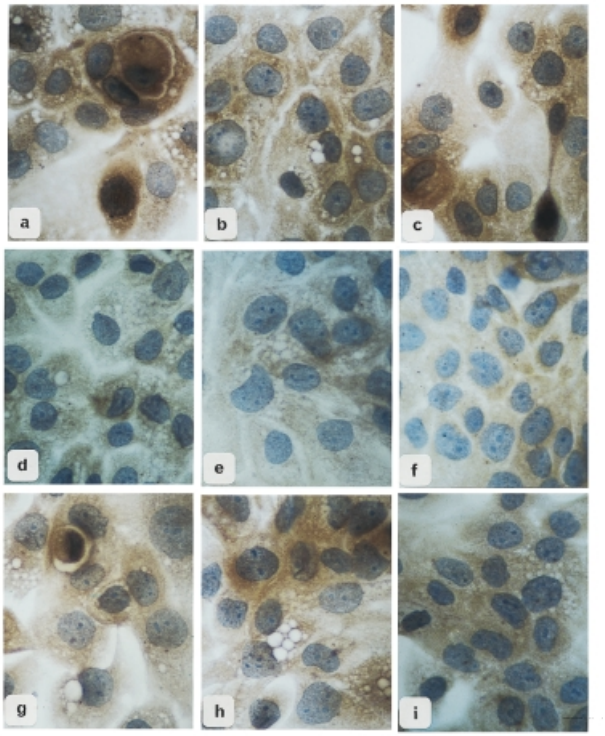

Fig. 3 Immunocytochemical Staining of MMP-1, MMP-8 and MMP-13.
In all three cell lines a cytoplasmic positive staining was seen for MMP-1 [HaCaT, (a); A5, (d); II-4RT, (g)] MMP-8 [HaCaT, (b); A5, (e); II-4RT, (h)] and MMP-13 [HaCaT, (c); A5, (f); II-4RT, (i)]. Magnification 340 x.

Table 2 Detection of MMP-1, -8, -13 and TIMP-1 in Extracts and Conditioned Media on Western Blots.

	Extracts				Conditioned media			
	HaCaT	A5	II-4RT	Form	HaCaT	A5	II-4RT	Form
MMP-1	+	+	+	latent	+	–	+++	latent
MMP-8	–	–	–	–	++	(+)	+++	active
MMP-13	+	+	+	latent	–	–	–	–
	+	+	+	active				
TIMP-1	+	+++	++	–	–	–	–	–

The intensities of the bands were scored from +++ (strong signal) to – (no signal).

Table 3 Scoring of the Immunocytochemical Staining.

	HaCaT	A5	II-4RT
MMP-1	12	12	12
MMP-8	3	6	7.5
MMP-13	8	8	8

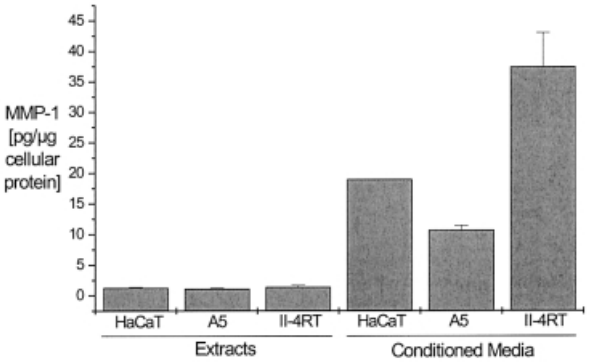

Fig. 4 Quantification of MMP-1 in Extracts and Conditioned Media of HaCaT, A5, and II-4RT Cells.
MMP-1 was quantified using the Amersham Biotrak MMP-1 ELISA kit. Values represent mean ± SD.

Immunocytochemistry The immunocytochemical analysis revealed the presence of immunoreactive MMP-1, -8 and -13 in all three cell lines, although to a different degree. The enzymes were localized intracytoplasmatically in a granular pattern. There was no accentuated membrane staining. Using a scoring system (see also Table 3) for the evaluation of the amounts of protein, we found equally intense stainings for MMP-1 and -13 in HaCaT (Figure 3a, g), A5 (Figure 3b, h) and II-4RT cells (Figure 3c, i). In contrast, the MMP-8 staining score was lower in HaCaT cells (Figure 3d) compared to the two tumorigenic cell clones – the benign A5 cells (Figure 3e) and the malignant II-4RT cells (Figure 3f) – which showed only minor differences.

ELISA The amounts of MMP-1 protein in the cytoplasm of the three cell lines were quantified by MMP-1-ELISA. As shown in Figure 4 the amounts of MMP-1 protein in HaCaT, A5 and II-4RT were virtually identical (1.3, 1.1, and 1.4 pg/µg of cellular protein, respectively).

Detection of Collagenases and TIMP-1 in Conditioned Media

Western Blot In contrast to the analysis of cytoplasmic collagenases-1 and -2 the pattern of secreted MMP-1 and MMP-8 differed significantly between malignant II-4RT cells and the other two cell lines (Figure 2B and 2C): while MMP-1 could not be found in media of benign A5 cells (Figure 2B, lane 4), it was present in small amounts in those of normal HaCaT cells (Figure 2B, lane 3) and enhanced in media of malignant II-4RT cells (lane 5). Secreted MMP-8 was visible as a 44 kDa band of active MMP-8. It was present in media of HaCaT (Figure 2C, lane 3) and A5 cells (hardly visible in Figure 2C, lane 4) and severalfold elevated in those of malignant II-4RT cells (Figure 2C, lane 5) (see also Table 2).

To verify whether the MMPs detected by Western blotting represent the latent or the active forms, purified MMP-1 and -8 with and without APMA treatment were used as controls. The 52 kDa band of the cell culture supernatants comigrated with the 52 kDa band of the control (MMP-1 without APMA treatment; Figure 2B, lane 1), indicating the presence of latent MMP-1 in the samples. In addition also active MMP-1 (44-41 kDa) was visible in the samples not treated with APMA (Figure 2B, lane 1). The 52 kDa band almost completely disappeared upon treatment of MMP-1 with APMA, so that only the bands of active MMP-1 (44-41 kDa) were visible (Figure 2B, lane 2). Application of MMP-8 controls (latent form: Figure 2C, lane 1; after APMA activation: Figure 2C, lane 2) demonstrated that the 44 kDa band corresponded to the active form of neutrophil collagenase (see also Table 2).

TIMP-1 and MMP-13 were not detectable in the conditioned media of any of the cell lines.

ELISA The enhanced secretion of MMP-1 by the malignant cells was confirmed and quantified by ELISA. As shown in Figure 4, HaCaT cell culture supernatants contained 19 pg/µg, those of benign A5 10.7 pg/µg and of malignant II-4RT 36 pg/µg of secreted protein.

Zymography In addition to the latent form of MMP-1 (52 kDa) the active enzyme (41 kDa) was found in conditioned media of benign A5 (Figure 5, lane 3) and malignant II-4RT (Figure 5, lane 4) cells by means of zymography. Comparison of the band pattern with that of MMP-1 controls clearly demonstrated that the lytic zones at 52 and 41 kDa correspond to the latent and active forms of MMP-1 (Figure 5, lane 1). The amount of active interstitial collagenase is very low and obviously below the detection limit of the less sensitive Western blot technique. In addition to the bands corresponding to MMP-1 also lytic bands at 60 and 56 kDa were obtained for all cell lines (Figure 5) which were not further characterized in this study.

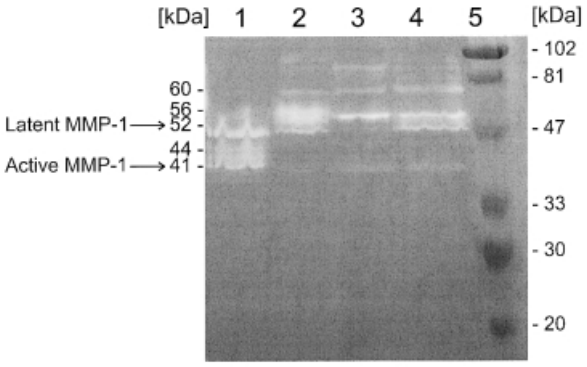

Fig. 5 Casein-Zymography of Conditioned Media.
Lane 1, MMP-1 control; lanes 2 – 4, conditioned media of HaCaT, A5 and II-4RT cells (20 µg protein per lane); lane 5, marker proteins.

Discussion

The dissolution of the extracellular matrix is a prerequisite for invasive malignant growth. Recent studies provide a large body of evidence that particularly matrix metalloproteinases (MMPs) are involved in this tissue degradation. Based on their structures and substrate specificities the MMPs have been classified into membrane-bound MMPs (MT-MMPs), which are mainly involved in the activation of non-bound MMPs, gelatinases (MMP-2, -9), which degrade non-helical collagen and non-collagenous proteins, stromelysins (MMP-3, -7, -10, -11), which mainly dissolve glycoproteins, and collagenases (MMP-1, -8, -13), which are the only mammalian enzymes that are able to cleave fibrillar collagens.

This substrate diversity is assumed to have major biological implications, particularly during the invasive growth of malignant tumors. Thus, the cleavage of the basement membrane as the first barrier for malignant epithelial cells by gelatinases and stromelysins needs to be followed by the breakdown of other interstitial collagen by collagenases. The production and activation of collagenases therefore is pivotal for further tumor growth and local tumor expansion. Indeed, various studies provide evidence that *in vivo* an enhanced production of interstitial collagenases is associated with a more aggressive growth potential and poor clinical outcome (Murray *et al.*, 1996, 1998; Cazorla *et al.*, 1998; Airola *et al.*, 1999; McCarthy *et al.*, 1999).

In a previous study (Bachmeier *et al.*, 1998; 2000) we have shown that HaCaT keratinocytes and the derived clonal cell lines of different tumorigenicity represent an interesting *in vitro* model for studying the biological role of the expression and secretion of gelatinases and stromelysins: the malignant cell clone (termed II-4RT, derived from the parental cell line HaCaT by c-Ha-ras oncogene transfection, Boukamp *et al.*, 1990) produces larger amounts of these enzymes than the benign cell line A5 and particulary the parental non-tumorigenic cell line HaCaT. This protease profile correlates well with the biological behaviour of these cell lines that has been extensively characterized by implantation studies in athymic mice: injection of HaCaT cells resulted in rapidly regressing nodules, A5 cells formed slowly expanding cysts without signs of invasive growth, while II-4RT cells produced locally invasive squamous cell carcinomas (Boukamp *et al.*, 1990). Thus, the enhanced production and secretion of gelatinases and to some extend of stromelysins in the tumorigenic clones may result in the degradation of type IV collagen and other components of the basement membrane.

In the present report we extended our studies on this model system to the analysis of the collagenases MMP-1, MMP-8 and MMP-13. These three collagenases are of special interest because they are able to degrade fibrillar collagens in the extracellular space, making them susceptible to further degradation by other MMPs. Thus, secretion of the collagenases enables tumor cells to invade the interstitial matrix.

Using RT-PCR all three cell lines were shown to express the mRNAs of MMP-1, -8, -13 and of TIMP-1, and thus are capable to synthesize the corresponding proteins. On the protein level, MMP-1 was significantly increased in the media of malignant cells when compared to the benign A5 and the non-tumorigenic HaCaT. So far the expression of this enzyme in HaCaT keratinocytes has not been described; however, several authors have reported the presence of MMP-1 in primary cultures of human squamous cell carcinomas (Petersen *et al.*, 1987; Bailly *et al.*, 1990). Interestingly, in addition to the latent form of MMP-1 also the active enzyme was found in the media of tumorigenic cell lines as detected by zymography, indicating that this collagenase may be activated by the tumor cells themselves even without the support of stromal cells. The intracellular levels of MMP-1 were about equal in the three cell lines as shown by ELISA, immunocytochemistry and Western blotting. The higher proportion of MMP-1 in media of the malignant II-4RT cells suggests that production and secretion of MMP-1 in II-4RT cells are enhanced in comparison to A5 and HaCaT cells and are linked to the malignant infiltrative behaviour.

In addition to MMP-1, MMP-8 was detected in all cell lines. The occurrence of this enzyme has so far not been reported in epithelial tumor cell lines, but was found in the granules of neutrophil leukocytes (Murphy *et al.*, 1977; Mainardi *et al.*, 1991) and also in other non-epithelial cells such as rheumatoid synovial fibroblasts, endothelial cells (Hanemaaijer *et al.*, 1997) and human chondrocytes (Chubinskaya *et al.*, 1996; Cole *et al.*, 1996). Cell-associated MMP-8 was identified in all three cell lines by immunocytochemistry, but not by Western blotting, most likely due to the lower sensitivity of this technique. MMP-8 was found on immunoblots of conditioned media of HaCaT and II-4RT; larger amounts are secreted by the malignant II-4RT than by non-tumorigenic HaCaT, while no MMP-8 was seen in media of A5 cells. As MMP-8 is known to hydrolyze native type I collagen with high efficiency (Hanemaaijer *et al.*, 1997), the secretion of this enzyme may be important for the invasive behavior of tumor cells.

MMP-13 was observed exclusively in cellular extracts as evidenced by Western blotting and by immunocytochemistry, but not in conditioned media. This is well in line with the previous report by Johansson *et al.* (1997b) who did not find MMP-13 in the media of unstimulated HaCaT cells; however, they reported that stimulation by TNF-α and TGF-β resulted in a release of the enzyme into the media. *In vivo* studies on various tumors revealed MMP-13 to be expressed by the epithelial tumor cells (Johansson *et al.*, 1997a; Cazorla *et al.*, 1998; Balbin *et al.*, 1999); in these cases the expression of MMP-13 may be due to stimulation by cytokines released *e. g.* by stromal cells.

The overall increased secretion of collagenolytic activity by II-4RT as compared to HaCaT and A5 cells seems to be reflected in the *in vivo* behavior of the cells: while the parental, non-tumorigenic HaCaT and the benign A5 cells apparently do not destroy their surrounding interstitial matrix *in vivo*, the malignant II-4RT cells show local dissolu-

tion of the collagenous matrix (Boukamp *et al.*, 1990) and therefore require collagenolytic activity in order to grow invasively.

Finally, we analyzed the expression of TIMP-1, which is capable to inhibit most MMPs, particularly MMP-1 (Matrisian, 1990). Interestingly, this inhibitor was detected in extracts of all three cell lines but no secretion could be demonstrated. The highest cell-associated levels were found in the tumorigenic, benign A5 cells, intermediate in malignant II-4RT cells, and the lowest in the non-tumorigenic HaCaT cells. Thus the amounts of cellular TIMP do not correlate well with those of the intracellular or secreted MMPs. At present, the implication of these findings, particularly for the control of the collagenases, is unclear. This subject is further complicated by the observation that TIMPs are not only MMP-inhibitors, but are also involved in protease activation (Strongin *et al.*, 1995).

Taken together, the results presented support the concept that the profile of MMPs synthesized and secreted by tumor cells correlates with the invasive phenotype of the respective cells. The increased constitutive secretion of MMP-1 and -8 by the malignant II-4RT cells appears to be an important molecular tool for the degradation of the extracellular matrix. Secretion and subsequent activation of MMP-13 seems to require the interaction with stromal cells, since this collagenase is found only in conditioned media of TNF-α / TGF-β stimulated but not of unstimulated HaCaT keratinocytes (Johansson *et al.*, 1997b). Surprisingly, the cell lines not only synthesize the collagenases MMP-1 and MMP-13, which are well known to be tumor-associated, but also the neutrophil collagenase MMP-8. Finally, the results confirm the conclusion of our accompanying study (Bachmeier *et. al.*, 2000) that the system of the three keratinocyte cell lines can serve as a highly valuable model for investigating correlations between the tumorigenic phenotype of cells and the expression of tumor-associated constituents.

Materials and Methods

Cells and Culture Conditions

Cell Culture Conditions The keratinocyte cell lines HaCaT, A5 and II-4RT (Boukamp *et al.*, 1988, 1990) were cultured at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. The cells were grown in DMEM (1 g/l glucose) supplemented with 10 % heat inactivated fetal calf serum, 0.35 mg/ml L-glutamine and 0.05 mg/ml gentamycin sulfate; for A5 and II-4RT cells gentamycine sulfate was substituted by 0.2 mg/ml geneticine. The medium was changed every three days. For subcultures cells were harvested after brief treatment with 0.1 % trypsin/EDTA solution and seeded at a dilution of 1:10. Cells between passages 12 and 55 were used for studies.

Preparation of Serum-Free Conditioned Medium Cells were grown to approx. 90 % confluence in 75 cm^2 plastic culture flasks containing 15 ml medium. The cultures were rinsed three times with Ca^{2+}- and Mg^{2+}-free PBS and subsequently 10 ml of serum-free medium was added. After 2 days, the conditioned medium was collected, concentrated by ultrafiltration (exclusion limit 10 kDa), and stored at –20 °C.

Harvesting of Cells Cells grown to confluence in serum-containing medium were washed three times with 5 ml PBS and harvested by scraping in 5 ml PBS. The cell supensions were centrifuged and the cells washed twice by suspension with 50 ml PBS and centrifugation (10 min at 300 *g*). Aliquots of 1.5 – 1.7×10^7 cells were stored at –70 °C until analysis.

Preparation of Cell Extracts 50 µl lysis buffer (10 mM Na_3PO_4; 0.4 M NaCl; 0.2 % Triton X-100) was added to an aliquot of frozen cells. The mixture was sonified and after centrifugation for 14 min at 15 000 *g* the supernatant containing the soluble proteins was collected and either analyzed immediately or stored at –20 °C.

Determination of Protein Concentration Protein concentrations were determined by the BCA protein assay (Pierce, Oud-Beijerland, Netherlands) with bovine serum albumin as standard.

Zymography

Electrophoresis was carried out under non-reducing conditions. After renaturation of the proteins by incubation of the gels in 25 g/l Triton X-100 at room temperature for 2 × 10 min they were incubated in 50 mM Tris-HCl, pH 7.5, containing 0.2 M NaCl, 0.02 % Brij35 and 10 mM $CaCl_2$ at 37 °C for 18 h. The gels were stained with Coomassie Brilliant Blue R-250. After destaining zones of proteolytic activity became visible as transparent bands in the blue gel.

As controls MMP-1 from human rheumatoid synovial fibroblasts (Calbiochem, La Jolla, USA) and MMP-8 from human neutrophil granulocytes (Calbiochem) were used. Rabbit muscle phosphorylase b, bovine serum albumin, ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor, hen egg lysozyme (Bio-Rad prestained markers, low range) were used as molecular mass markers.

Reverse Transcription and Polymerase Chain Reaction

RNA Extraction Total RNA was extracted from cells according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) using TRI Reagent (Sigma, Deisenhofen, Germany). Total RNA was quantified at 260/280 nm. Aliquots were stored in DEPC-treated water or 0.1 M sodium acetate at –80 °C.

Reverse Transcription cDNAs were synthesized from 1 µg of total RNA using the specific outer reverse primers (Table 1). Reactions were carried out with the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Freiburg, Germany) following the manufacturer's instructions.

Semi-Nested PCR The first round of PCR was carried out with 1/10 of the synthesized specific cDNA, 10 pmol of each primer, 40 nmol dNTP, 1 U PANScript polymerase (PAN-Systems), 2.5 µl 10x-buffer [16 mM $(NH_4)_2SO_4$, 50 mM Tris/HCl, 0.1 g/l Tween-20, pH 8.8] in a total volume of 25 µl. The $MgCl_2$ concentration in the reaction mixture was 6 mM for MMP-1, 2 mM for MMP-8 and -13, and 3 mM for TIMP-1.

20 (MMP-1) or 25 (MMP-8, -13 and TIMP-1) cycles were carried out with 30 s denaturation (94 °C), 30 s annealing (56 °C), and 30 s (MMP-8, TIMP-1) or 1 min (MMP-1, MMP-13) synthesis (72 °C).

The second round of PCR with the forward and inner reverse primers was carried out using one µl of the first PCR mixture in a total volume of 50 µl and 35 PCR cycles. Concentrations of all reagents and cycle profiles were as in the first PCR.

Negative controls were carried out in the same manner but without template.

Characterization of RT-PCR Products PCR products were characterized by restriction fragment analyses and size determination using polyacrylamide gel electrophoresis in 14 % gels containing 10 % glycerol.

Immunoblotting

Sixty µg protein of cell extract or 15 µg protein of conditioned media were subjected to SDS-PAGE in 10 % polyacrylamide slab gels under non-reducing conditions. After electrotransfer to a nitrocellulose membrane and blocking with 50 g/l low fat dry milk powder in TTBS (50 mM Tris-base, 150 mM NaCl, pH 7.5, 1 g/l Tween-20) the membranes were incubated for 2 h in polyclonal rabbit antiserum (for anti-MMP-1 see Lichtinghagen *et al.*, 1995; for anti-TIMP-1 see Geisler *et al.*, 1997; anti-MMP-8 and -13 were supplied by Dr. Harald Tschesche, Bielefeld), washed three times for 10 min in TTBS, incubated for 1 h with a 1:1000 dilution of porcine anti-rabbit immunoglobulin G alkaline phosphatase conjugate (DAKO) in 10 g/l low fat dry milk powder in TTBS. After rinsing twice with TTBS and once with TBS (TTBS without Tween), the membrane was incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium to visualize bands of alkaline phosphatase activity.

Molecular mass marker proteins and purified MMP-1 (150 ng; Calbiochem) and -8 (100 ng; Calbiochem) were used as controls; in some experiments the purified MMPs were activated with APMA prior to electrophoresis.

ELISA

MMP-1 was quantified in extracts and conditioned media from different passages of the three cell lines using a MMP-1-ELISA Kit (Biotrak, Amersham Buchler, Braunschweig, Germany). The antibody used in this ELISA recognizes the zymogen of MMP-1, the active form and the complex with TIMP-1.

Immunocytochemistry

For the immunocytochemical localization of MMP-1, -8 and -13 cells were grown to confluence on silanized sterile glass slides (SuperFrost plus). The medium was discarded, the cells rinsed with Tris buffer, fixed in methanol/acetone (v/v 2:1) for two minutes, and rinsed again. Following incubation with the specific polyclonal primary antibodies (see 'immunoblotting') for 30 minutes (37 °C), rinsing with Tris buffer, and application of the secondary antibody system (Streptavidin-Biotin-Complex method, Hsu and Raine, 1981; DAKO, Hamburg, Germany), the resulting antibody complexes were visualized with diaminobenzidine (Sigma).

Using light microscopy the proportion of positively labeled cells and the staining intensity was quantified. A five step grading score was used for the proportion of stained cells (score 0: 0 cells stained; score 1: 0 – 25 cells stained; score 2: 26 – 50 cells stained, score 3: 51 – 75 cells stained; score 4: >75 cells stained) and a four step grading for the staining intensity (score 0: no positive staining; score 1: faint positive staining; score 2: moderate staining; score 3: strong staining intensity). 10 randomly selected areas (each 0.41 mm^2) on two different slides of each cell line were evaluated.

This score was calculated by multiplication of the amount of positively labeled cells and the staining intensity.

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