Expression of membrane type 1 matrix metalloproteinase in papillomavirus-positive cells: role of the human papillomavirus (HPV) 16 and HPV8 E7 gene products

Sigrun Smola-Hess,1 Jenny Pahne,1 Cornelia Mauch,2 Paola Zigrino,2 Hans Smola2† and Herbert J. Pfister1

1Institute of Virology, University of Cologne, Fürst-Pückler-Str. 56, 50935 Cologne, Germany
2Department of Dermatology, University of Cologne, Joseph-Stelzmann-Str. 9, 50924 Cologne, Germany

Matrix metalloproteinases (MMPs) degrade extracellular matrix. They are involved in cellular proliferation, migration, angiogenesis, invasion and metastasis. MT-1 MMP, a membrane-bound MMP, is expressed in carcinomas of the uterine cervix in vivo. This type of cancer is associated with human papillomavirus (HPV) infection. Here it was shown that keratinocytes transformed with HPV16 or HPV18 in vitro, and HPV-positive cervical carcinoma cell lines, constitutively expressed MT-1 MMP. Expression of the E7 protein from the mucosal and cutaneous high-risk types HPV16 and HPV8, but not from the cutaneous low-risk type HPV1, was sufficient to induce MT-1 MMP expression in primary human keratinocytes and HaCaT cells. As a consequence, MMP-2 was activated. MT-1 MMP expression might play a role in the HPV life cycle by promoting proliferation of host cells and might contribute to their invasive phenotype during malignant progression.

Persistent infection with genital high-risk human papillomavirus (HPV) types, e.g. HPV16 or HPV18, contributes to the development of genital cancers, which are characterized by deregulated, high-level expression of the HPV early genes E6 and E7 (reviewed by zur Hausen, 2002). In 99.7% of established cervical carcinomas, papillomavirus DNA has been detected (Walboomers et al., 1999). In patients suffering from the rare genetic skin disease epidermodysplasia verruciformis (EV), HPVs have been detected in benign skin tumours as well as in squamous cell carcinomas (SCCs) (Fuchs & Pfister, 1996). These HPV types were subsequently designated EV-associated HPVs. Moreover, seroreactivity to the cutaneous EV-associated HPV8 is correlated with a significantly higher risk for epithelial skin cancers, even in non-EV-diseased, immunocompetent individuals (Feltkamp et al., 2003; Masini et al., 2003).

Malignant tumour cells have acquired mechanisms to degrade basement membrane structures and invade the underlying tissue. Tumour invasion finally determines the prognosis of the disease. Production of the matrix-degrading type IV collagenase matrix metalloproteinase (MMP)-9 and activation of MMP-2 have been correlated to metastatic disease progression (Azzam et al., 1993; Liotta et al., 1980; Nuovo et al., 1995). These so-called gelatinases are produced by the tumour cells and by surrounding stromal cells (Okada et al., 1995). In contrast to these enzymes, collagenase-2 (MMP-8) may have an opposite role in carcinogenesis. Loss of MMP-8 even conferred increased skin tumour susceptibility to male mice (Balbin et al., 2003). MMP-9 is regulated by many cytokines at the transcriptional level. We have recently shown that cervical carcinoma cells do not constitutively produce MMP-9; however, this protease can be induced by CD40 ligand (Smola-Hess et al., 2001). In HPV16-transgenic mice, MMP-9 is mainly localized in haematopoietic cells within the tumour stroma (Coussens et al., 2000). Pro-MMP-2, the zymogen of MMP-2, is constitutively produced in most cell types (reviewed by Ries & Petrides, 1995). Total MMP activity in the tissue is regulated by complex post-translational mechanisms like proteolytic conversion and activation of the zymogen, interaction with extracellular matrix components and regulation by tissue inhibitors of metalloproteinases (TIMPs) (Goldberg et al., 1989). MMP-2 activation is of particular interest for tumour cell invasion. This MMP has been extensively studied in pre-neoplastic and malignant lesions of the uterine cervix. It could be shown that MMP-2 expression and activation gradually increased during progression and correlated with poor prognosis (Brummer et al., 2002; Davidson et al., 1999; Gaiotto et al., 2004; Sheu et al., 2003). However, MMP-2,

†Present address: Paul Hartmann AG, PO Box 1420, D-89504 Heidenheim, Germany.
MT-1 MMP expression has recently been detected in cervical cancer specimens in vivo and invasiveness has been correlated with high-level expression of MT-1 MMP (Gilles et al., 1996; Sheu et al., 2003). However, it remains unclear whether MT-1 MMP is constitutively produced in HPV-positive cells or induced by environmental stimuli comparable to the MMP-9 expression pattern (Smola-Hess et al., 2001). In this study, we first analysed MT-1 MMP expression in HPV-transformed keratinocytes and cervical carcinoma cell lines in vitro and then investigated the impact of HPV on MT-1 MMP expression in HaCaT cells, an HPV-negative cell line, and in primary human keratinocytes.

To study MT-1 MMP expression, the following cell lines were analysed: SiHa, CaSkI (both HPV16-positive), the HPV18-positive SCC lines C4-1 and SW756 and adeno-carcinoma cell line HeLa, as well as keratinocytes transformed in vitro with HPV16 (HPKIA; Durst et al., 1987) or HPV18 (K5-1). Total RNA was extracted from monolayer cell cultures using the RNAzol B (WAK-Chemie Medical) according to the manufacturer’s instructions. Total RNA (20 μg) of each sample was analysed by Northern blot hybridization with a random-primed 32P-labelled cDNA probe for MT-1 MMP (kindly provided by Dr H. Sato, Knasawa University, School of Medicine, Knasawa, Japan) and subsequently with an 18S rRNA probe. All investigated cell lines expressed the 4.5 kb MT-1 MMP transcript constitutively (Fig. 1) except the adeno-carcinoma cell line HeLa, which has been shown to be negative for MT-1 MMP (Okada et al., 1995). These data demonstrated that MT-1 MMP expression is constitutive, at least in the HPV-positive SCCs, and, unlike pro-MMP-9, does not require further exogenous activation. Interestingly, MT-1 MMP was also strongly expressed in non-malignant keratinocytes transformed with genital high-risk HPV. This suggested that the virus itself might contribute to MT-1 MMP expression, even in non-malignant keratinocytes.

To test for a functional relationship between early viral genes and MT-1 MMP induction, we first transfected HPV-negative HaCaT keratinocytes with expression plasmids for E7. Investigating the function of the E7 oncogene has the problem that E7 can display pro-apoptotic activity if no E6 protein is present, which can overcome p53-mediated cell death (Howes et al., 1994). Thus, it is theoretically possible that cells stably expressing E7 undergo compensatory changes that are subsequently attributed to the E7 protein itself. HaCaT cells appear to be especially suitable for analyses of E7 functions, since these cells carry mutated alleles of p53 (Boukamp et al., 1988; Lehman et al., 1993). It has been demonstrated that HaCaT cells retrovirally infected with HPV16 E7 expression constructs even show a significant reduction in their apoptotic response to genotoxic stress (Magal et al., 1998). Moreover, HaCaT keratinocytes only express low levels of baseline MT-1 MMP and have previously been successfully used for other functional studies of HPV early proteins (Kabsch & Alonso, 2002; Matthews et al., 2003). For construction of an HPV16 E7 expression plasmid (pcDNA-16E7), an NsiI–KpnI fragment of HPV16 (nt 257–884) was ligated into the pcDNA3.1+ expression vector (under the control of the cytomegalovirus promoter) within HindIII and KpnI sites using an appropriate adapter. HPV8 E7 was excised from pZipneo-HPV8E7 (Ifnner et al., 1988) with BamHI, cloned into pBS SK II+ and then directionally inserted into pcDNA3.1+ resulting in pcDNA-8E7. HaCaT cells were stably transfected with 10 μg Scal-linearized pcDNA-16E7, pcDNA-8E7 or the empty vector pcDNA3.1+ as a mock control using Lipofectamine (Invitrogen). After selection with G418 (Invitrogen), neomycin-resistant clones were tested for the genomic presence of HPV16 E7 by PCR and for HPV16 E7 mRNA expression by RT-PCR (data not shown). Three clones expressing HPV16 E7 were compared with nine vector-transfected mock clones to exclude positional effects of the transgene.

![MT-1 MMP mRNA](image_url)
Mock-transfected clones expressed only low levels of MT-1 MMP mRNA, while for HaCaT clones expressing the HPV16 E7 gene product much stronger hybridization signals were observed, slightly varying among the clones analysed (Fig. 2a, upper left panel). To test MT-1 MMP protein expression levels in the E7 transfectants, we prepared membranes from all cell clones by ultracentrifugation on a 38% sucrose cushion (model L565; Beckman Instruments) for 1 h at 100,000 g. MT-1 MMP protein was detected by Western blot analysis with anti MT-1 MMP mAb (2 μg ml⁻¹; Calbiochem) followed by detection with peroxidase-labelled goat anti-mouse Ab and the ECL substrate reaction (Amersham Pharmacia Biotech) following the manufacturer’s instructions (Fig. 2b). MT-1 MMP protein was expressed at higher levels in the HPV16 E7-transfected clones than in the mock-transfected controls, corresponding to the mRNA expression pattern. Furthermore, MT-1 MMP mRNA and protein were not only upregulated in HPV16 E7-expressing but also in HPV8 E7-expressing HaCaT clones (Fig. 2a and b, right panels).

In order to confirm the data obtained with the keratinocyte cell line HaCaT in primary cells, human foreskin keratinocytes (Cambrex Bio Science) were infected with the pLXSN expression vector (BD Biosciences Clontech) or pLXSN-HPV16E7 (ATCC)-containing retroviruses. The coding sequences of HPV8 E7 and HPV1 E7 were cloned into EcoRI and BamHI or XhoI sites, respectively. Infected primary keratinocytes were selected in keratinocyte basal culture medium (Cambrex Bio Science) containing 100 μg G418 ml⁻¹. Total RNA was extracted and subjected to Northern blot analysis as described above. Whereas the E7 proteins from HPV16 and HPV8 were able to upregulate MT-1 MMP expression (Fig. 2c, left panel), in HPV1 E7-expressing keratinocytes MT-1 MMP mRNA expression levels were similar to the pLXSN vector control cells (Fig. 2c, right panel). These data suggested that the E7 protein is also sufficient to induce MT-1 MMP expression in primary human keratinocytes. MT-1 MMP induction was not restricted to cells expressing the E7 protein derived from a mucosal HPV but was also observed with a cutaneous high-risk HPV type. In contrast, the E7 protein derived from the cutaneous low-risk type HPV1 was not able to upregulate MT-1 MMP.

This finding is interesting since it might explain high MT-1 MMP expression not only in carcinomas of the uterine cervix but also in papillomas, dysplastic skin and SCCs of mice transgenic for the early genes of HPV8 under the control of the cytokeratin 14 promoter (H. J. Pfister & C. Mauch, unpublished observations). Thus, the E7 protein from high-risk HPV's was able to induce MT-1 MMP in the context of the whole virus (HPKIA cells), the entire HPV early region (HPV8-transgenic mice) and in a p53-mutated

Fig. 2. MT-1 MMP expression in HaCaT transfectants at the mRNA and protein levels. (a) Total RNA (20 μg) from HaCaT clones 1–9 transfected with pcDNA3.1+ vector alone, pcDNA-16E7 clones 1–3 or pcDNA-8E7 (clones 1 and 2) was subjected to Northern blot analysis and probed for MT-1 MMP mRNA expression. 18S rRNA is shown in the lower panel. (b) Membranes of the transfectants and a subset of mock transfectants (corresponding to clones 7–9) were prepared and tested for MT-1 MMP protein by Western blot analysis. (c) MT-1 MMP mRNA expression in retrovirally infected primary human foreskin keratinocytes. Total RNA from keratinocytes retrovirally infected with pLXSN control vector or pLXSN-1E7, -8E7 or -16E7, as indicated, was subjected to Northern blot analysis and probed as in (a). Scans were performed using SnapScan e50 and the SCANWISE software.
keratinocyte cell line, as well as in primary human keratinocytes.

We next studied whether MT-1 MMP expression in the E7-transfected HaCaT clones led to activation of pro-MMP-2. HaCaT wild-type, mock and stably E7-transfected HaCaT keratinocytes were seeded in 24-well plates at a density of $3 \times 10^5$ cells per well in DMEM/10% fetal calf serum. After 20 h, cells were washed twice with serum-free DMEM and incubated for 24 h in 200 µl serum-free DMEM. To assess constitutive versus inducible MMP activity in zymograms, the cells were also stimulated with different concentrations of TNF-α in serum-free medium. This inflammatory cytokine is a product of activated macrophages, which are present in the infiltrate of cervical carcinoma in vivo. TNF-α is known to be a potent inducer of gelatinase activity in many different cell types. For zymograms, 20 µl of the conditioned media was separated by 10% SDS-PAGE containing 1 mg gelatin (Sigma) ml$^{-1}$. The gels were washed in 2.5% Triton X-100 for 30 min, followed by incubation at 37°C for 48 h in 50 mM Tris/HCl pH 8.0 and 5 mM CaCl$_2$ and were finally stained with Coomassie blue R250 (Merck) to reveal bands with gelatinolytic activity.

As shown in Fig. 3 HaCaT control cells and all transfected HaCaT clones constitutively produced the 72 kDa pro-form of MMP-2. Addition of increasing doses of TNF-α gradually induced the 92 kDa form of MMP-9 but had only marginal effects on MMP-2 activation. A striking difference between HPV E7 and mock transfectants was noted concerning MMP-2 activation. Neither HaCaT wild-type cells nor mock transfectants produced significant amounts of activated MMP-2 (Fig. 3, upper panel). In contrast, in supernatants of HaCaT cells expressing HPV16 E7 (Fig. 3, middle panel) and also HPV8 E7 (Fig. 3, lower panel) the processed 62 and 59 kDa forms of MMP-2 were present, indicating proteolytic activation. Thus, MMP-2 was constitutively activated in the E7-expressing keratinocytes and did not require further stimulation by pro-inflammatory cytokines. TNF-α only slightly enhanced E7-mediated pro-MMP-2 activation at the highest concentrations.

Among tumour viruses, only the viral hepatitis B virus X protein has been demonstrated to induce MT-1 MMP expression and MMP-2 activation utilizing a cyclooxygenase-2-dependent mechanism (Lara-Pezzi et al., 2002). We therefore investigated the effect of the highly specific and potent cyclooxygenase-2 inhibitor PTPBS (Calbiochem; IC$_{50}$ = 32 nM) on E7-expressing HaCaT cells. Even at a concentration higher than 10 µM, this inhibitor affected neither MT-1 MMP mRNA expression levels nor MMP-2 activation (data not shown), indicating that E7 might utilize a pathway that is distinct from the hepatitis B virus X protein. Further analyses will have to elucidate whether E7 regulates MT-1 MMP expression directly or whether secondary cellular mechanisms, like induction of autocrine signalling mechanisms, might be employed.

---

**Fig. 3.** Pro-MMP-2 activation in E7-expressing HaCaT clones. HaCaT wild-type cells and mock transfectants (upper panel), and HPV16 E7-expressing (middle panel) and HPV8 E7-expressing (lower panel) HaCaT clones were seeded at equal densities ($3 \times 10^5$ cells per well), reconfirmed by a neutral red uptake assay. Cells were stimulated with serum-free medium or TNF-α (in serum-free medium) at $10^{-1} - 10^3$ U ml$^{-1}$. Twenty-four hours later, the supernatants were harvested and gelatin zymography was performed. Arrows indicate gelatinolytic activities at 92 kDa corresponding to pro-MMP-9, at 72 kDa corresponding to the pro-form of MMP-2, and at 62 and 59 kDa corresponding to activated forms of MMP-2. Scans were performed using SnapScan e50 and the SCANWISE software.
What could be the role of MT-1 MMP upregulation in HPV-positive keratinocytes? Obviously, MT-1 MMP expression plays a major role in pro-MMP-2 activation and therefore provides a basis for the cells to acquire an invasive phenotype. However, this is not the only function of MT-1 MMP. Upregulation of this protease may also have a physiological role during the viral life cycle by promoting cellular proliferation. Recently, a list of novel substrates for MT-1 MMP has been reported, which includes MT-1 MMP itself, plasminogen, chemokines, cytokines and a sheddase activity for the transmembrane mucin MUC1, playing a role in tumour progression (Teske et al., 2004; Thathiah & Carson, 2004; for review see Sternlicht & Werb, 2001). Plasminogen induces selective proteolysis of insulin-like growth factor (IGF)-binding protein-4 (IGFBP-4) and promotes autocrine IGF-II bioavailability in human carcinoma cells (Remacle-Bonnet et al., 1997). IGF can promote keratinocyte proliferation, a prerequisite for HPV replication (Aaronson et al., 1990). It has also been shown that MT-1 MMP cleaves CD44 and the γ2 chain of laminin 5, all associated with cell migration (Kajita et al., 2001; Koshikawa et al., 2000), thus enhancing the autonomous locomotion of cells. In addition, MT-1 MMP seems to enhance angiogenesis through upregulation of vascular endothelial growth factor expression (Sounni et al., 2002), which is observed in pre-malignant stages of HPV-induced intraepithelial lesions and in HPV-associated cancer (Dobbs et al., 1997; Guidi et al., 1995; Obermair et al., 1997; Smith-McCune et al., 1997).

In summary, our data demonstrate that the E7 proteins of the high-risk HPV types 16 and 8 lead to the induction of MT-1 MMP, possibly explaining high MT-1 MMP expression in HPV8-transgenic mice (unpublished). This E7-mediated mechanism may profoundly alter the phenotype of infected keratinocytes, providing a basis for cellular proliferation, angiogenesis and also, later in malignant progression, for invasion of HPV-positive tumour cells.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft through the DFG-Forscherguppe (Az FOR 265/2-1) and the SFB 589 at the University of Cologne to S.-S.-H. (and also to C. M.) S.-S.-H. has been supported by the Heisenberg programme of the DFG. The authors are grateful to U. Sandaradura de Silva for excellent technical assistance. TNF-α was a kind gift from Dr G. R. Adolf, Bender & Co., Vienna, Austria. The MT-1 MMP probe was kindly provided by Dr H. Sato, Knazawa University, School of Medicine, Knazawa, Japan.

References


Sabe, F., Ota, I., Holmbeck, K. & 10 other authors (2004). Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP. J Cell Biol 167, 769–781.


