Spontaneous tumour development in human papillomavirus type 8 E6 transgenic mice and rapid induction by UV-light exposure and wounding

Gian Paolo Marcuzzi,1,2 Martin Hufbauer,1 Hans Udo Kasper,3 Sönke Jan Weißenborn,1 Sigrun Smola2,4 and Herbert Pfister1,2

1Institute of Virology, University of Cologne, 50935 Cologne, Germany
2Center for Molecular Medicine Cologne, University of Cologne, 50931 Cologne, Germany
3Department of Pathology, University of Cologne, 50924 Cologne, Germany
4Institute of Virology, Saarland University, 66421 Homburg/Saar, Germany

Correspondence
Gian Paolo Marcuzzi
gian.marcuzzi@uk-koeln.de

Cutaneous human papillomavirus type 8 (HPV8) is carcinogenic in patients with epidermodysplasia verruciformis. Transgenic mice with the complete early region (CER) of HPV8 spontaneously developed papillomas, dysplasia and squamous cell carcinomas of the skin. To characterize the role of individual early genes in carcinogenesis, the E6 and E6/E7 genes were expressed separately in transgenic mice. Nearly all HPV8-E6-positive mice spontaneously developed multifocal tumours, characterized by papillomatosis, hyperkeratosis and varying degrees of epidermal dysplasia. In 6% of the cases, the tumours became malignant, comparable with HPV8-CER mice. Thus, in the murine epidermis, E6 is the major oncogene necessary and sufficient to induce spontaneous tumour development up to the level of squamous cell carcinoma. To evaluate the synergistic effects of UV light and wound healing, the skin of HPV8 mice was irradiated with UVA/UVB light or wounded with punch biopsies. These treatments induced papillomatosis in HPV8-CER and -E6 mice within 3 weeks. Irradiation with UVA alone did not induce papillomatosis and UVB alone had a weaker effect than UVA/UVB, indicating a synergistic role of UVA in UVB-induced papillomatosis. An HPV8 infection persisting over decades in interaction with sun burns and wound healing processes may be a relevant cause of skin cancer in humans.

INTRODUCTION

Human papillomaviruses (HPVs) belong to a large family of small DNA viruses and are able to induce hyperproliferations in cutaneous and mucosal epithelia that extend from benign to pre-malignant and malignant lesions. Mucosal high-risk HPV types are designated carcinogens in the anogenital tract and the oropharynx (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2007), but the role of cutaneous HPV types from the genus Betapapillomavirus in skin carcinogenesis is still a matter of debate. There is compelling evidence for the carcinogenicity of HPV5 and HPV8 in patients with epidermodysplasia verruciformis (EV), a rare, autosomal-recessive, cancer-prone genetic disorder (Orth, 2006). These patients develop characteristic flat warts and macular lesions during childhood, which are induced by at least 17 HPV types (betapapillomaviruses). Later in life, squamous cell carcinomas (SCCs) arise, mostly in sun-exposed skin areas. They are associated with a subset from the genus Betapapillomavirus, mostly HPV5 and to a lesser extent HPV8, -17, -20 and -47 (Orth, 2005). Epidemiological studies have shown that betapapillomaviruses are not limited to EV patients. They are frequently detected in swabs of healthy skin and in plucked eyebrow hairs of the general population (de Koning et al., 2009; Weißenborn et al., 2009). They have been identified in more than 50% of non-melanoma skin cancers (NMSCs) of immunocompetent individuals (Asgari et al., 2008) and in up to 90% of cutaneous SCCs of immunosuppressed organ transplant recipients (Pfister, 2003). The betapapillomavirus types 5 and 8 do not dominate in NMSCs of non-EV patients.

Expression of the complete early genome region (CER) of HPV8 in transgenic mice has been shown to be sufficient for the spontaneous development of NMSC (Schaper et al., 2005). Transgenic mice expressing only the HPV8 early gene E2 present infundibular hyperplasia and acanthosis combined with mild to severe dysplasia, mostly in their second year of life (Pfefferle et al., 2008). In E2-expressing
mice, tumours did not show the prominent papillomatosis and hyperkeratosis seen in HPV8-CER mice, and the tumour incidence was about twofold lower and tumour development about twice as long.

Extensive studies of the mucosa-associated HPV types (e.g. HPV16) in vitro and in transgenic mice have shown that the oncoproteins E6 and E7 are the most important for cancer development by inhibiting regulation of the cell cycle and apoptosis (Herber et al., 1996; Song et al., 1999; zur Hausen, 2000).

In order to characterize the role of HPV8 E6 and E7 in carcinogenesis, we generated transgenic mice that expressed the E6 gene individually or in combination with E7. The HPV8 E6 and E6/E7 genes were under the control of the keratin-14 promoter, which activates expression of these genes in basal keratinocytes.

The role of sunlight in the aetiology of NMSC is well known (Dahle et al., 2005; Durham et al., 2003; Sander et al., 2003). The main risk factors for cutaneous SCC are prolonged sun exposure and pigmentation grade of the skin. Interestingly, several studies have revealed diverse interactions of persisting betapapillomaviruses and UV irradiation, which may be relevant to the multi-step process of skin carcinogenesis (Akgil et al., 2005; Giamperi & Storey, 2004; Jackson & Storey, 2000; Jackson et al., 2002; Underbrink et al., 2008). We therefore evaluated the synergistic effects of UV light in transgenic animals.

METHODS

Construction and preparation of the transgene. The HPV8 E6 and E6/E7 genes were amplified by PCR and inserted into the BamHI site of the K14CreERTom plasmid under the control of the human keratin-14 promoter (Vasioukhin et al., 1999). The BamHI site is located between the sequence of the second intron of the rabbit β-globin gene and the K14 polyadenylation sequence. The recombinant vectors K14-HPV8-E6 and K14-HPV8-E6/E7 were digested with HindIII and SacI flanking the K14 expression cassette. The appropriate fragment was purified via agarose gel electrophoresis and Millipore membrane filtration.

Generation of transgenic mice. The linearized transgenes were microinjected into the pro-nucleus of fertilized DBA/BL6-F2 oocytes (Harlan Laboratories), which were implanted into pseudopregnant surrogate mothers as described by Hammes & Schedl (2000) to produce putative founder mice expressing the HPV8 E6 or E6/E7 transgenes. Transgene-positive mice were back-crossed into FVB/N wild-type mice. Genomic DNA was isolated from tail biopsies of 3-month-old mice using a QIAmp Tissue kit (Qiagen). Samples of wild-type mice. Genomic DNA was isolated from tail biopsies of 3-month-old mice using a QIAmp Tissue kit (Qiagen). Samples of

Quantification of mRNA. RNA from skin biopsies was isolated with an RNeasy kit and DNase digestion was performed on a column using RNase-free DNase according to the manufacturer’s instructions (Qiagen). One microgram of RNA was reverse transcribed using an Omniscript kit (Qiagen) with 1 μM oligo(dT)20 primer (Sigma), 10 μM random nonamers (TIB MOLBIOL) and 10 U RNase inhibitor (Fermentas).

Hyperoxanthine phosphoribosyltransferase 1 (HPRT1) and HPV8 E6 expression levels were measured by real-time PCR using a LightCycler System (Roche). Two microtiter of a 1:10 dilution of cDNA were used in a total volume of 20 μl containing 1.25 U Platinum Taq polymerase and associated buffer (Invitrogen), 4 mM MgCl2, 1.6 μl SYBR Green (1:1000 dilution; Sigma), 5% DMSO, 0.5 μM each forward and reverse primer (see Supplementary Table S1), 500 ng non-acetylated BSA (Fermentas) μl-1 and 0.2 mM each dNTP. Samples were analysed in duplicate, together with an appropriate plasmid or cDNA dilution series, which was used to generate a standard curve. Mean values were used for the calculation of expression ratios of HPV8 E6 and the housekeeping gene HPRT1. The cycling protocol conditions were 60 s at 95 °C, followed by 40 cycles of 1 s at 95 °C (20 °C s-1), 5 s at the relevant annealing temperature (20 °C s-1) and 15 s at 72 °C (20 °C s-1). Fluorescence was measured once per cycle at the end of the elongation step.

Analysis of H-ras status in mouse DNA from skin or tumour biopsies. Exons 2 and 3 of H-ras from mouse DNA were amplified and sequenced. Amplifications were performed in 50 μl with 1 U Taq DNA polymerase (Fermentas) and associated buffer containing 200 μM each dNTP, 0.3 μM each primer (see Supplementary Table S1), 1.5 mM MgCl2 and 2 μl template DNA. Cycling conditions were 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 1.5 min at 57 °C and 2 min at 72 °C, with a final elongation step for 10 min at 72 °C.

UV-light exposure. UV radiation (UVR) was generated by a UV device (UV 801; Waldmann) equipped with eight PUVA lamps (UVA: 320–400 nm) and four UV21 lamps (UVB: 280–360 nm with a peak at about 314 nm). The minimal erythemal dose (MED) was determined at 0.5 J cm-2 for UVB and 10 J cm-2 for UVA. The energy output of the UV device as measured at the level of the skin surface using a radiometer was 1.41 mW cm-2 for the UVR region and 6.34 mW cm-2 for the UVA region (UV Radiometer Variocontrol; Waldmann). The administered doses were 0.1, 0.25, 0.5 and 1 MED for UVA and 0.5, 1 and 2 MED for UVB. Prior to UVR exposure, mice were anaesthetized and their back area was shaved with an electric shaver. A 4 cm2 area was irradiated while the rest of the skin was covered with a UVR-impermeable sheet.

Wounding experiments. For the wounding experiments, the animals were anaesthetized before shaving of the back. Four punch biopsies were taken from the skin of the back creating four circular 4 mm wounds.

Statistical analyses. Statistical analyses were carried out using SPSS v14 (SPSS).

RESULTS

Generation of transgenic mice. Transgenic mice were generated that expressed the HPV8 E6 gene individually or in combination with E7 under the control of the human keratin-14 promoter, which activated total RNA preparation were stored at −20 °C in RNALater according to the manufacturer’s protocol (Qiagen). The district government of Cologne approved the generation of the transgenic mice (50.203.2 K 29, 33/01) and the UV irradiation experiments (50.203.2 K 29, 23/04).
expression of the transgenes in the basal keratinocytes. Transgenic animals were backcrossed with FVB/N mice, as previous studies have shown the susceptibility of the FVB/N inbred mouse strain to epidermal carcinogenesis (Woodworth et al., 2004). This is due to a polymorphism in the Pch1 gene, which promotes SCC formation (Wakabayashi et al., 2007). We obtained seven HPV8-E6-positive founder mice. From three different animals, lines were established with up to five generations (nos 631, 635 and 637). All of the transgene-positive mice of line 635 were male. Thus, the transgene appeared to be integrated into the Y chromosome. All E6 transgenic mouse lines were heterozygous for the transgene. No line could be established from two female HPV8-E6/E7-positive founder mice, because all of the offspring were transgene negative.

### Development of spontaneous skin tumours in HPV8-E6 and -E6/E7 transgenic mice

The HPV8-E6 and -E6/E7 transgene-positive F0 mice spontaneously developed skin tumours at the age of 8–21 weeks. Tumour growth generally started in the dorsocranial region of the trunk with alopecia and hyperkeratosis, and continued to become more hyperkeratotic and spread diffusely (30%) or multifocally (70%) to the caudal and ventral regions of the body (Fig. 1). The macroscopic appearance and the localization of the skin lesions in the HPV8-E6 mice were almost identical to previously described skin tumours in HPV8-CER mice (Schaper et al., 2005). Skin lesions continuously progressed and never regressed. Altogether, 95/98 transgenic mice (97%) from the F0 up to the F3 generation developed tumours when they were observed for at least 51 weeks. None of the HPV8-E6-negative littermates developed skin tumours within an observation period of up to 85 weeks. The only recognizable macroscopic difference between the HPV8-E6 and HPV8-CER transgenic mice was the development of thymus tumours, which occurred in 72% of the HPV8-E6 mice with skin lesions. The size of the thymus tumours varied from a slight enlargement of 0.4 cm diameter to a greatly increased diameter (1.5 cm × 1.5 cm × 1 cm). In healthy mice, the thymus has a size of approximately 0.2 cm × 0.2 cm. None of six examined HPV8-E6 mice without any skin lesions at the age of 8–12 weeks showed abnormal thymus growth.

The time course of tumour development in the three transgenic mouse lines (631, 635 and 637) is shown as Kaplan–Meier curves in Fig. 2. Positive mice that died due to unknown reasons before they developed a tumour were not included. The transgenic HPV8-E6 mice of the F1 generation of lines 631 and 637 developed skin lesions after 12 and 14 weeks, and mice of line 635 after 25 weeks. In all lines, the mice of the F2 generation developed tumours about 7–9 weeks earlier than mice of the F1 generation.

Altogether, 47 HPV8-E6 transgenic mice with tumours were analysed histologically. The lesions were characterized by papillomatosis, hyperkeratosis, dyskeratosis and para-

keratosis. Cavities formed inside the epidermis, which were filled with lamella-like, concentric keratin masses (Fig. 3a). Twenty-six of the examined tumours (55%) were classified as papillomas without dysplasia. Papillomas with different degrees of dysplasia were diagnosed for 18 tumours (38%). A mild dysplasia was seen in six tumours (13%; Fig. 3b). They showed atypical changes in the basal layer with an increased number of mitotic cells, moderate hyperchromatosis and anisonucleosis. In three cases (6%) with severe dysplasia, the layering of the epidermis was no longer present. The cells were strongly atypical with hyperchromatic and pleomorphic nuclei, but the basal membrane was still intact (Fig. 3c). In three cases (6%), SCCs with infiltrative growth of the tumour cells into the connective tissue were observed (Fig. 3d, insert). In one of these cases, the tumour cells had even invaded the bone of the skull (Fig. 3d, arrows). Skin tumours that developed in the two HPV8-E6/E7-positive founder mice both showed signs of moderate or severe dysplasia. The histological analyses are summarized in Table 1.

As H-ras mutations are almost always found in mouse skin tumours after chemical carcinogenesis, we sequenced exons 2 and 3 of the H-ras gene from HPV8-E6-induced papillomas and carcinomas. No H-ras mutations were found in six papillomas (two each with low, moderate and severe dysplasia) and two SCCs.
To increase the number of SCCs, we also analysed ten cancers of HPV8-CER mice. Two separate tumour compartments were tested in each case. A point mutation from CAA to CTA in codon 61 was detected in both samples of two cancers and in one sample each of two additional cancers. Altogether, H-ras mutations were only found in 6/22 SCC samples from HPV8-E6 and HPV8-CER mice and thus are clearly less prevalent than in the context of chemical carcinogenesis. Interestingly, the H-ras status turned out not to be homogeneous throughout two cancers.

**UV irradiation of HPV8-CER and -E6 mice rapidly induces papillomatosis**

To simulate the situation of sunburn early in human life, we irradiated HPV8-CER-positive and -negative mice at 4 weeks of age, when they do not show any skin abnormalities. The mice were irradiated with a solar-simulated spectrum comprising 10 J cm\(^{-2}\) UVA (320–400 nm) and 1 J cm\(^{-2}\) UVB (280–360 nm), which is an inflammatory dose that causes sunburn cell formation. A single treatment with UV light was sufficient to induce papillomatosis in all HPV8-CER FVB/N mice after 3 weeks. In contrast, the irradiated skin of the HPV8-negative littermates healed completely within that time (Fig. 4). The minimal dose for induction of papillomatosis was 2.5 J cm\(^{-2}\) UVA and 0.25 J cm\(^{-2}\) UVB. UVB irradiation only (0.36 J cm\(^{-2}\)) was sufficient to induce a few solitary papillomas after 6 weeks within the treated area. A single irradiation with 10 J cm\(^{-2}\) UVA had no effect, even after 2 months of observation. Histology revealed no differences between UV-induced and spontaneous tumours, which appeared later on non-irradiated skin. UV-irradiated HPV8-CER C57/BL6 mice from the F\(_5\) generation
developed papillomas within the treated area as early as HPV8-CER FVB/N transgenics. The same rate of papilloma development was observed with UV-irradiated HPV8-E6 mice (Table 2).

UV irradiation of HPV8-CER and -E6 mice enhances HPV8-E6 mRNA expression

The level of E6 mRNA may play an important role in HPV8-induced papilloma development. To compare HPV8-CER and HPV8-E6 mice, the levels of E6 mRNA were measured in duplicate by quantitative (q)RT-PCR in RNA from shaved skin biopsies from three mice each. The E6 results were normalized to the mRNA levels of the housekeeping gene HPRT1 in these biopsies. Similar HPV8 E6:HPRT1 ratios were detected in HPV8-E6 mice (0.29 ± 0.11) and HPV8-CER mice (0.65 ± 0.41).

To investigate whether UV irradiation can enhance HPV8 E6 expression and thereby contribute to the rapid induction of papilloma development, six HPV8-CER mice and three HPV8-E6 mice were UV irradiated. Skin biopsies were taken 4 and 12 days after UV irradiation from HPV8-CER mice and 12 days after UV irradiation from HPV8-E6 mice. Control biopsies were taken from non-irradiated skin areas 12 days after UV irradiation. HPV8 E6 and HPRT1 mRNA levels were measured by qRT-PCR. Because HPRT1 mRNA levels were increased after UV irradiation of the skin, this gene could not be used for normalization. UV induction was even stronger in the case of β-actin, which was tested as an alternative reference gene. Therefore, the HPV8 E6 mRNA levels were normalized to the RNA input of 1 μg. In HPV8-CER mice, the E6 mRNA levels were enhanced about 18-fold by 4 days after UV irradiation and rose to about 27-fold after 12 days. In HPV8-E6 mice, the E6 mRNA levels were enhanced about 12-fold by 12 days after UV irradiation (Fig. 5).

Wounding experiments

Due to our observations that the skin lesions arose spontaneously mostly in places where the transgenic mice

Table 1. Tumour histology in three HPV8-E6 mouse lines and from F0 mice without progeny

<table>
<thead>
<tr>
<th>Histological change</th>
<th>631 FVB/N</th>
<th>635 FVB/N</th>
<th>637 FVB/N</th>
<th>F0 mice (without progeny)</th>
<th>All lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papilloma</td>
<td>14</td>
<td>5</td>
<td>6</td>
<td>1 (E6)</td>
<td>26</td>
</tr>
<tr>
<td>Papilloma with mild dysplasia</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>5</td>
<td>3</td>
<td>–</td>
<td>1 (E6/E7)</td>
<td>9</td>
</tr>
<tr>
<td>Severe dysplasia</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>1 (E6)</td>
<td>3</td>
</tr>
<tr>
<td>SCC</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>11</td>
<td>9</td>
<td>4</td>
<td>47</td>
</tr>
</tbody>
</table>
scratched themselves, we wanted to examine whether tissue damage was sufficient to induce papillomas or whether DNA damage by UV light is necessary for tumorigenesis. The skin was wounded by taking punch biopsies 4 mm in diameter in 4-week-old mice. After approximately 10 days, the wounds healed in the HPV8-negative littermates, whereas the HPV8-positive mice showed signs of papillomatosis. After 17 days, the skin lesions were already clearly visible and were massive after 27 days (Fig. 6). Overall, the four wounds of 14 HPV8-positive animals all gave rise to

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**Table 2.** Induction of papillomatosis with different UVA/UVB doses in HPV8-CER and -E6 transgenic mice

<table>
<thead>
<tr>
<th>HPV8-CER, -E6 pos. (+) or neg. (−)</th>
<th>UV dose (J UVA/UVB cm⁻²)</th>
<th>Age (days)</th>
<th>n</th>
<th>Presence of tumours</th>
<th>FVB/N or BL6</th>
</tr>
</thead>
<tbody>
<tr>
<td>CER⁺</td>
<td>1/0.1</td>
<td>37</td>
<td>3</td>
<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁻</td>
<td>1/0.1</td>
<td>37</td>
<td>1</td>
<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁺</td>
<td>2.5/0.25</td>
<td>37</td>
<td>6</td>
<td>5</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁻</td>
<td>2.5/0.25</td>
<td>37</td>
<td>2</td>
<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁺</td>
<td>5/0.5</td>
<td>70</td>
<td>3</td>
<td>3</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁻</td>
<td>5/0.5</td>
<td>70</td>
<td>1</td>
<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁺</td>
<td>10/1</td>
<td>24</td>
<td>12</td>
<td>12</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁻</td>
<td>10/1</td>
<td>24</td>
<td>4</td>
<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁺</td>
<td>10/1</td>
<td>56</td>
<td>9</td>
<td>9</td>
<td>BL6</td>
</tr>
<tr>
<td>CER⁻</td>
<td>10/1</td>
<td>56</td>
<td>3</td>
<td>0</td>
<td>BL6</td>
</tr>
<tr>
<td>CER⁺</td>
<td>0.36 UVB</td>
<td>28</td>
<td>6</td>
<td>6</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁻</td>
<td>0.36 UVB</td>
<td>28</td>
<td>2</td>
<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁺</td>
<td>10 UV A</td>
<td>28</td>
<td>6</td>
<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>CER⁻</td>
<td>10 UV A</td>
<td>28</td>
<td>2</td>
<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>E6⁺</td>
<td>5/0.5</td>
<td>46</td>
<td>3</td>
<td>3</td>
<td>FVB/N</td>
</tr>
<tr>
<td>E6⁻</td>
<td>5/0.5</td>
<td>46</td>
<td>1</td>
<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>E6⁺</td>
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<td>37</td>
<td>12</td>
<td>12</td>
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<td>E6⁻</td>
<td>10/1</td>
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<td>0</td>
<td>FVB/N</td>
</tr>
<tr>
<td>E6⁺</td>
<td>0.36 UVB</td>
<td>71</td>
<td>3</td>
<td>3</td>
<td>FVB/N</td>
</tr>
<tr>
<td>E6⁻</td>
<td>0.36 UVB</td>
<td>71</td>
<td>1</td>
<td>0</td>
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</tr>
</tbody>
</table>
hyperplasia (Carraresi et al., 1990; Bailleul et al., 1992). Those H-ras mutations were the result of an interaction of the carcinogen with the DNA. Therefore, H-ras activation is an early event in chemical- but not in HPV8-induced carcinogenesis. About 10–20% of human SCCs have mutations in codons 12, 13 and 61 of H-ras (Boukamp, 2005).

HPV8-positive and -negative mice were irradiated with different doses of UVA/UVB to simulate the situation of sunburn in humans. The mean MED in Caucasians is 240 J m \(^{-2}\), whereas for 1–5% of the population MED is about 50 J m \(^{-2}\). The carcinogenic potential of sunburn is not limited to high MEDs, since patients with skin lesions frequently sustain MEDs below 100 J m \(^{-2}\). Nevertheless, the major carcinogenic effect is the greatest for the largest MEDs, which is why the MEDs in the mice were increased stepwise (Fig. 5).

DISCUSSION

The HPV8-E6 transgenic mice generated in this study showed quite similar E6 mRNA levels to the previously described HPV8-CER transgenics. They represented an almost exact phenocopy of the HPV8-CER mice regarding frequency and time course of tumour development, as well as general appearance of the skin tumours. In view of the fact that five out of seven HPV8-E6-positive mice of the F0 generation developed tumours later in life, the possibility of tumour induction by insertional mutagenesis is negligible. Histological analysis showed no substantial differences between the tumours of HPV8-E6 and HPV8-CER transgenic mice. In both cases, papillomas were the most frequent tumour type, followed by papillomas with low and moderate dysplasia. Papillomas with severe dysplasia and SCC arose in both transgenic mouse strains at a frequency of 3 and 6%, respectively. SCCs were diagnosed in all three HPV8-E6 lines.

The only remarkable difference between HPV8-E6 and HPV8-CER transgenic animals was the occurrence of thymus hyperplasia in 72% of the HPV8-E6-positive mice with skin lesions. It has been shown that the endogenous keratin-14 gene and some K14 promoter-driven transgenic constructs are expressed in the cortical epithelium of the thymus gland (Capone et al., 2001; Laufer et al., 1996; McGargill et al., 2000). HPV16-E6/E7 transgenic mice under the control of the keratin-5 promoter, which directs the expression to basal epithelial cells, all developed thymus hyperplasia (Carraresi et al., 2001). The absence of thymus hyperplasias in HPV8-CER mice may be due to the expression of additional viral genes.

The two HPV8-E6/E7 transgenic animals both developed skin tumours with a high degree of dysplasia. However, on the basis of only two animals, it would be premature to speculate that E7 promotes tumour progression, particularly as this cannot be supported by observations in HPV8-CER mice, which also express E7.

The comparison with HPV8-CER transgenics showed that E6 is the major oncogene of HPV8 in the murine epidermis. It is necessary and sufficient to induce spontaneous tumour development up to the level of SCC. Similarly, skin tumours in HPV16-E6 transgenic mice frequently became malignant, as opposed to tumours in HPV16-E7 mice, which were mostly benign (Song et al., 1999).

The strong oncogenic activity of the HPV8 E6 protein is remarkable in view of major deficits in capacities regarded as relevant in carcinogenesis. In contrast with HPV16 (Scheffner et al., 1990), the E6 proteins of the EV-associated HPV5 and HPV8 do not interact with p53 and do not degrade p53 (Elbel et al., 1997; Kiyono et al., 1994; Steger & Pfister, 1992). HPV16 E6 shows a strong or moderate interaction with the cellular proteins E6AP and NFX1-91, respectively, which are known to be important for telomerase activation. HPV8 E6 interacts very weakly with E6AP and not at all with NFX1-91 (Bedard et al., 2008). In line with the relative strength of these protein–protein interactions, HPV8 E6 induces only modest telomerase levels in human foreskin keratinocytes, approximately 20% of that observed with HPV16 E6. Thus, crucial oncogenic mechanisms of HPV8 E6 probably remain to be elucidated.

In 40% of the examined SCCs of HPV8-CER transgenic mice, a mutation in codon 61 of the H-ras gene could be identified, which leads to constant activation of ras-mediated signal transduction. In the other 60%, this activation does not seem to have been the cause for the progression up to SCC. In none of the benign lesions could H-ras mutations be detected. These results are in contrast to the data from chemical skin carcinogenesis mouse models with dimethylbenzanthracene and 12-O-tetradecanoylphorbol-13-acetate, in which 95–100% of the papillomas harboured mutations in codon 61 of H-ras (Andrews et al., 1990; Bailleul et al., 1989; Gill et al., 1992). Those H-ras mutations were the result of an interaction of the carcinogen with the DNA. Therefore, H-ras activation is an early event in chemical- but not in HPV8-induced carcinogenesis. About 10–20% of human SCCs have mutations in codons 12, 13 and 61 of H-ras (Boukamp, 2005).

HPV8-positive and -negative mice were irradiated with different doses of UVA/UVB to simulate the situation of sunburn in humans. The mean MED in Caucasians is within the order of 5 J solar-simulated UV light cm \(^{-2}\),...
which consists of about 4.5 J UVA cm\(^{-2}\) and 0.5 J UVB cm\(^{-2}\) (Halliday & Lyons, 2008). In HPV8-transgenic FVB/N and BL6 mice, a single dose in the range of the MED in Caucasians was sufficient to induce papillomatosis within 3 weeks. The time interval between irradiation and papilloma development was almost the same in both genetic backgrounds. A UVB dose of 0.36 J cm\(^{-2}\) was a weak inductor of papillomatosis. However, a lower UVB dose together with UVA induced the development of papillomas over the entire irradiated surface. Thus, UVA and UVB seem to act synergistically in the induction of papillomatosis. Papilloma growth may be related to the UV-induced increased expression of HPV8 genes, which could be measured by 4 days after irradiation. In the mouse model, the increased expression is triggered by the UV-inducible K14 promoter (Horio et al., 1993; Smith & Rees, 1994). Regarding the natural conditions of infected humans, it is noteworthy that UVB irradiation induces the promoter of HPV5 and HPV8 (Akgül et al., 2005).

HPV8-CER and -E6 transgenic animals wounded with punch biopsies developed papillomas at the wound sites in 100% of the cases. This clearly demonstrates that the DNA damage caused by UV is not a necessary prerequisite for tumorigenesis in our mouse model. The expression of HPV8 E6 started to increase by 1 day after wounding (data not shown), which is consistent with increasing levels of K14 expression following skin wounding (Werner & Munz, 2000). Epidermal repair in humans with extensive burns is frequently associated with the generation of anti-HPV5 antibodies (Favre et al., 2000). The presence of antibodies may reflect activation of viral gene expression finally

**Fig. 6.** HPV8-CER-positive FVB/N mice and negative littermates were wounded at the age of 4 weeks with 4 mm punch biopsies (as shown above). After 4 days, an eschar was visible on both positive and negative mice. After 10 days, the wounds had healed well in the negative animal, but in the positive animal these changed slowly into papillomas. After 17 days, the induction of papillomatosis was pronounced in the positive mouse and prominent after 27 days.
leading to the synthesis of capsid proteins. Interestingly, in all papillomas, irrespective of whether they arose spontaneously or whether they were induced by UV light or skin wounding, a strong stromal inflammatory infiltrate was observed (data not shown). In other skin tumour models, it has previously been demonstrated that inflammatory responses play an important role in neoplastic progression (CousSENS et al., 1999). At the molecular level, it was shown that chronic inflammation promoting tumorigenesis in the skin involves an interaction between the receptor RAGE and its putative ligands, the calgranulins S100A8 and S100A9 (GEBHARDT et al., 2008). It will therefore be interesting to analyse further the nature of the inflammatory response in the HPV8 transgenic mice.

The rapid initiation of HPV8-driven tumorigenesis in the mouse skin by wounding following punch biopsies or UV light is certainly related to the strong expression of HPV8 in all proliferation-competent keratinocytes under the control of the K14 promoter. Expression of the early genes of betapapillomaviruses from the viral promoter in basal epidermal layers during natural infection in humans usually remains below the detection limits of in situ hybridization or real-time RT-PCR (DANG et al., 2006; HALLER et al., 2001). However, even a low-level infection may contribute to skin carcinogenesis when persisting over decades in interaction with sunburns and wound-healing processes.

REFERENCES


