Upregulation of lipocalin-2 in human papillomavirus-positive keratinocytes and cutaneous squamous cell carcinomas

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It has been demonstrated previously that E7 oncogene expression of human papillomavirus (HPV) type 8 in keratinocytes induces cell invasion and accelerated differentiation. Looking for cellular genes deregulated by HPV-8 E7, lipocalin-2 was identified as being upregulated in these cells by cDNA microarray analysis. Lipocalin-2 is known to be overexpressed in many human cancers and is implicated in the regulation of cell proliferation, differentiation and apoptosis. In this study, increased levels of lipocalin-2 were observed in extracts from HPV-8 E7-positive keratinocytes and from keratinocytes expressing E7 of HPV-1, -4, -5, -15, -20 and -38, but not of HPV-16. Similar results were obtained when measuring secreted lipocalin-2 in the supernatants of the cell cultures. Lipocalin-2 expression was associated with cell differentiation in keratinocyte monolayers and in organotypic skin cultures. It was found in the uppermost layers of HPV-5, -8, -15, -16, -20 and 38 E7-expressing but not low-risk HPV-1 and -4 E7-expressing keratinocytes. Immunohistochemical staining of HPV-positive and -negative human skin squamous cell carcinomas (SCCs) revealed lipocalin-2 expression mostly in differentiated, filaggrin-positive areas of 15 out of 17 HPV-positive and three out of nine HPV-negative SCCs. These data indicate that lipocalin-2 expression correlates with HPV positivity of cutaneous SCCs.

INTRODUCTION

Human papillomaviruses (HPVs) are small dsDNA viruses that infect keratinocytes and cause epithelial lesions of varying severity. Infection with a subset of high-risk mucosal α-HPV types (e.g. HPV-16 or -18) is the major risk factor for the development of cervical cancer (zur Hausen, 2002). Cutaneous HPVs display a much greater heterogeneity and belong to different genera based on nucleotide sequence comparisons and similar biological or pathogenic properties. The paradigm for a link between HPV infection and the development of cervical cancer is the rare genetic disease epidermodysplasia verruciformis (EV). EV patients are highly susceptible to HPV infection and develop cutaneous squamous cell carcinomas (SCCs). In contrast to the diversity of β-PV genotypes in benign skin lesions, only a subset of β-PVs, most frequently HPV-5 and occasionally HPV-8, -14, -17, -19, -20, -24 or -47, are associated with malignant conversion to SCCs in EV patients (Dell’Oste et al., 2009; Orth, 2006). As in EV patients, the risk of developing cutaneous SCC is markedly increased in immunosuppressed patients, and β-HPV DNA has frequently been detected in actinic keratoses and skin cancers of organ transplant recipients. By studying the seroreactivity to β-HPVs and the presence of β-HPV DNA in actinic keratoses and cutaneous SCCs, recent epidemiological studies have also suggested a role for β-HPVs in the pathogenesis of SCCs in the immunocompetent general population (Bouwes Bavinck et al., 2008).

Whilst cutaneous and mucosal HPVs encode similar early-region proteins that can participate in lesional development, it is now apparent that the cellular targets of the virally encoded proteins are different (reviewed by Akgül et al., 2006). In addition, whilst mucosal cancers typically contain at least one copy of the HPV genome per cell and viral gene expression is required to maintain the transformed phenotype, the HPV DNA load in cutaneous cancers is low, and one HPV copy can be detected in only 10–1000 dysplastic cells. The presence of HPV is obviously not mandatory for maintenance of the malignant phenotype of skin cancer cells. The highest prevalence rate of the
β-types is found in actinic keratoses, which suggests a particular involvement of HPVs in the early stages of cutaneous oncogenesis (Pfister et al., 2003; Weissenborn et al., 2005).

The mechanisms by which cutaneous HPVs contribute to the development of non-melanoma skin cancers are poorly understood at present. Using an organotypic system with primary human adult keratinocytes (PHAKs) cultured on human dermis, we have shown that expression of the HPV-8 E7 gene induced accelerated differentiation and promoted invasion of keratinocytes into the dermis (Akgül et al., 2005). Using this organotypic system, expression of the E7 genes of HPV-1 (μ-type), HPV-4 (γ-type), HPV-5, -8, -15, -20 and -38 (β-types) and the mucosal HPV-16 (α-type) showed different effects on the expression of differentiation and proliferation markers in the regenerated epithelium (Westphal et al., 2009).

To unravel the molecules involved in the cellular transformation process induced by HPV-8, we employed cDNA microarray analysis of PHAKs infected with recombinant retroviruses encoding HPV-8 E7 and identified the gene encoding lipocalin-2 as being stimulated by HPV-8 E7. Human lipocalin-2 is a member of the lipocalin family, which comprises more than 50 known members, all of which are characterized by their low molecular mass. Lipocalins have been found to bind and transport small essential factors or to provide growth factor effects, thus modulating cellular responses such as differentiation and proliferation (Devarajan, 2007). Deregulated expression of lipocalin-2 has been reported in several malignant conditions. It is significantly upregulated in breast, lung, gastric, colon, ovary and pancreatic carcinomas (reviewed by Devarajan, 2007).

An association of lipocalin-2 expression with cutaneous HPV infection has not yet been explored. Here, we analysed the expression of lipocalin-2 in human keratinocytes in monolayer cultures as well as in organotypic skin cultures expressing HPV genes and in HPV-positive and -negative human cutaneous SCCs.

RESULTS

Lipocalin-2 is induced by HPV-8 E7 in primary adult keratinocytes

Analysis of the differentially expressed genes in PHAKs transduced with HPV-8 E7 revealed lipocalin-2 to be upregulated. To support the finding that lipocalin-2 might be a putative cellular target gene of HPV-8 E7, we analysed its protein expression by Western blotting in total cell extracts from PHAKs (from donor 1) retrovirally infected with empty retroviral vector pLXSN or pLXSN-8 E7. As shown in Fig. 1(a), lipocalin-2 protein levels were increased in PHAKs expressing HPV-8 E7 when compared with the control cell extracts, thus confirming its induction by HPV-8 E7.

Lipocalin-2 protein is expressed and secreted in keratinocytes expressing different HPV E7 genes

To confirm the overexpression of lipocalin-2 observed in the keratinocytes of donor 1, we characterized its expression levels in the keratinocytes of a second donor (donor 2) to exclude variation due to the specific genetic background of the cells. To see whether the induction of lipocalin-2 expression is commonly shared by E7 proteins
of HPVs, we also expressed the E7 genes of HPV-1, -4, -5, -15, -16, -20 and -38, types from different genera associated with different clinical manifestations. Western blot analysis of total cell extracts from these cells showed that the amount of lipocalin-2 protein was increased more than 2-fold by expression of HPV-15, -20 and -38 E7. We found moderate induction (1.5–1.8-fold) by HPV-1, -5 and -8 and no changes in lipocalin-2 levels in HPV-4 and -16 E7 extracts when compared with the control cells (Fig. 1b). As lipocalin-2 is a well-known secretory protein, we quantified its secreted levels in the supernatants of the keratinocyte cultures. Consistent with the Western blot data, ELISA analysis showed increased levels of lipocalin-2 in conditioned medium from HPV-20 (3.5-fold) and -38 (5-fold) and moderately increased amounts (2-fold) in medium from HPV-15 E7-transduced cells (Fig. 1c). The changes in lipocalin-2 secretion from HPV-1, -4, -5, -8 and -16 E7-transduced cells were not significant.

Lipocalin-2 is induced by keratinocyte differentiation

Under normal conditions, the maturation of cutaneous keratinocytes is linked to the differentiation programme of the skin. It has been demonstrated previously that lipocalin-2 is a calcium-inducible gene in human keratinocytes (Lee et al., 2008; Seo et al., 2004). To test whether lipocalin-2 expression changes in differentiating HPV-8 E7-expressing PHAKs, cells were exposed to high extracellular calcium (2 mM) for up to 7 days, which is known to induce cell differentiation. Western blots revealed upregulation of the differentiation marker involucrin, thus confirming the differentiation-inducing culture condition (Fig. 2a). Whereas differentiation increased the lipocalin-2 levels in all differentiating keratinocytes, a substantially higher level of lipocalin-2 was found in HPV-8 E7 cells at day 7 (Fig. 2a). However, although they showed more lipocalin-2 protein expression in Western blots, these cells did not secrete more lipocalin-2 when switched to high calcium (Fig. 2b). The comparison of HPV-8 E7 with HPV-20 and -38 E7, which showed considerable upregulation of lipocalin-2 in low calcium (Fig. 1b), revealed that, upon calcium induction, the relative fold increase in lipocalin-2 was higher in HPV-8 E7-positive (3.4-fold) than in HPV-20 E7-positive (1.8-fold) and HPV-38 E7-positive (1.3-fold) keratinocytes. Thus, together, these results indicated that calcium-induced keratinocyte differentiation is associated with an increase in cell-associated but not secreted lipocalin-2 in HPV E7-expressing cells.

Induction of lipocalin-2 in organotypic skin cultures of PHAKs expressing HPV E7 genes

To confirm the association of induced expression of lipocalin-2 and cell differentiation, we also analysed its expression in organotypic skin cultures. This in vitro culture of skin is a useful system for the analysis of skin biology because it mimics keratinocyte differentiation more effectively than monolayer cultures (Akgül et al., 2005; Westphal et al., 2009). Human keratinocytes were infected with HPV E7-containing and empty pLXSN-derived retrovirus, whose retroviral 5’LTR-promoter activity is insensitive to keratinocyte differentiation, and seeded onto de-epidermalized dermis repopulated with human fibroblasts as described in Methods. After 14 days in the air–liquid interface (conditions that induce epidermal differentiation), the raft cultures were paraffin-embedded and 4 μm sections were obtained and stained for lipocalin-2. As demonstrated in Fig. 3, lipocalin-2 was...
detected in the uppermost layers of the HPV-5, -8, -16, -20 and -38 E7-expressing but not the control and HPV-1 or -4 E7-expressing epithelia. These data suggested that lipocalin-2 expression is localized in layers of differentiating keratinocytes expressing E7 of oncogenic α- and β-HPV types but not of the wart-associated types HPV-1 and HPV-4.

Expression of lipocalin-2 is associated with HPV positivity in cutaneous SCCs

To evaluate the expression of lipocalin-2 in cutaneous SCCs, we performed an immunohistochemical study of a panel of human skin SCCs. Lipocalin-2 expression was examined in 17 HPV-positive and nine HPV-negative SCCs. We found 15 out of 17 (88%) HPV-positive and three out of nine (33%) HPV-negative tested SCCs that were positive for lipocalin-2 expression, indicating a significant difference in lipocalin-2 expression between HPV-positive and -negative SCCs (Fig. 4a). In HPV-positive tumour sections, lipocalin-2 was always found in differentiated tissue areas. Due to the fact that Mallbris et al. (2002) described lipocalin-2 expression in SCC areas lacking filaggrin, we performed co-immunostaining of HPV-positive SCCs for lipocalin-2 and filaggrin. Fig. 4(b) clearly shows that lipocalin-2 and filaggrin were co-expressed in the same cells, suggesting that lipocalin-2 is present in differentiated, filaggrin-positive areas of cutaneous SCCs (Fig. 4b).

DISCUSSION

The lipocalin-2 gene was found to be directly or indirectly upregulated by E7 of the β-HPV types 5, 8, 20 and 38, both in monolayer and in organotypic cultures of PHAKs. HPV-1 E7 induced lipocalin-2 expression only in monolayer cultures. In contrast, HPV-16 E7 was only able to induce lipocalin-2 expression upon keratinocyte differentiation in organotypic cultures. Lipocalin-2 was not detectable in organotypic cultures of skin keratinocytes devoid of HPV E7, in line with a previous study of normal human skin (Mallbris et al., 2002). However, strong induction of lipocalin-2 has been observed in a variety of skin disorders characterized by dysregulated epithelial differentiation. In psoriasis and pityriasis rubra, production of lipocalin-2 was confined to spatially distinct subpopulations of keratinocytes underlying areas of parakeratosis, whereas skin samples lacking parakeratotic epithelium such as lichen ruber planus, acute contact eczema and basal cell carcinoma were negative for lipocalin-2 (Mallbris et al., 2002; Seo et al., 2006). This strict link between lipocalin-2 expression and parakeratosis cannot be transferred to our findings in HPV E7-expressing organotypic skin cultures, as parakeratosis...
was exclusively observed with HPV-38 (Westphal et al., 2009).

The upregulation of lipocalin-2 by HPV-16 E7 is of interest in view of a recent report showing the expression of lipocalin-2 to be related to the presence and load of genital high-risk HPVs (Syrjänen et al., 2010). The expression of lipocalin-2 furthermore increased with the grade of cervical lesions.

We extended our observations from the cell culture experiments to human skin SCCs and found a significant proportion of HPV-positive SCCs with increased levels of lipocalin-2 compared with HPV-negative tumours. Given that cutaneous SCCs contain a low HPV load, this finding supports the hypothesis that persistent viral infection in a few keratinocytes may elicit a bystander effect on neighbouring cells. In the HPV-positive SCCs, immunostaining for lipocalin-2 was only distributed in the most differentiated layers of the transformed epithelium. Strong lipocalin-2 expression in differentiated keratinocytes of five cutaneous SCCs has been described previously (Mallbris et al., 2002; Seo et al., 2006) without testing for HPV. Lipocalin-2 expression could also be detected in differentiated nests of oesophageal SCCs (Zhang et al., 2007). Whereas Mallbris et al. (2002) described lipocalin-2 expression in SCC areas lacking filaggrin, we observed co-localization of filaggrin and lipocalin-2 in HPV-positive SCCs.

To appreciate the possible consequences of lipocalin-2 overexpression in HPV-positive tissues, it should be noted that knocking down lipocalin-2 expression by stable

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**Fig. 4.** (a) Immunohistochemical analysis of lipocalin-2 expression in HPV-negative and -positive skin SCC specimens. Tissue samples contained the indicated HPV types. Paraffin-embedded tissue sections of specimens were stained with anti-lipocalin-2 antibody (red) and counterstained with DAPI (blue). Magnification, ×200. (b) Co-immunostaining of an HPV-positive SCC sample for lipocalin-2 (red) and filaggrin (green). Magnification, ×200.
transfection of lipocalin-2-targeting small interfering RNA in the thyroid carcinoma FRO cells blocked the ability of the cells to grow in soft agar and to form tumours in nude mice. Decreasing lipocalin-2 expression significantly reduced the invasion and migration ability of HER2-positive breast cancer cells (Iannetti et al., 2008; Leng et al., 2009). This suggests a direct involvement of lipocalin-2 in tumorigenic processes in these tissues. Furthermore, lipocalin-2 is involved in cellular iron import and export and can thereby inhibit or induce apoptosis (Devireddy et al., 2005). On the one hand, lipocalin-2 may thus act as a survival factor for cancer cells, reducing apoptosis induced by pro-apoptotic agents (Iannetti et al., 2008; Tong et al., 2005). On the other hand, lipocalin-2-mediated export of iron and downregulation of ferritin may induce apoptosis mediated by the pro-apoptotic protein Bim (Devireddy et al., 2005). However, in our system, no differences in Bim, cleaved caspase-3 and poly(ADP–ribose) polymerase (PARP) expression were detected (data not shown). In spite of its potentially oncogenic functions, a relevant role of lipocalin-2 in skin tumour development remains questionable in view of the restricted expression in different keratinocytes. However, lipocalin-2 is regarded as a marker for dysregulated keratinocyte differentiation in human skin (Mallbris et al., 2002) and it is noteworthy that this type of dysregulation is preferentially observed in β-HPV-positive cutaneous SCCs. The precise biological effect of lipocalin-2 needs to be investigated in the future by RNA interference in HPV-expressing keratinocytes.

**METHODS**

**Retroviral constructs and generation of recombinant retroviruses.** The construction of pLXSN-8 E7 and generation of pLXSN-based E7 expression vectors for HPV-1, -4, -5, -16, -20 and -38 have been described previously (Akgül et al., 2005; Westphal et al., 2009). The production of recombinant retroviruses, subsequent transduction into PHAKs and selection of stable, 100%–transduced clones were performed as described previously (Akgül et al., 2005).

**Monolayer cell culture.** PHAKs from Caucasians (donors 1 and 2) were isolated from abdominal tissues, and isolated cells were subsequently passaged and maintained in defined keratinocyte serum-free medium (Invitrogen) containing 0.05 mM calcium. To obtain differentiated keratinocytes, cells were cultured for up to 7 days in medium containing 2 mM calcium.

**Organotypic skin cultures.** Organotypic cultures were carried out as described previously (Akgül et al., 2005). Briefly, glycerol-preserved skin (Euro Skin Bank, Beverwijk, The Netherlands) was washed three times in PBS and incubated in PBS containing antibiotics (600 U penicillin G ml⁻¹, 600 μg streptomycin sulfate ml⁻¹, 250 μg gentamicin sulfate ml⁻¹ and 2.5 μg Fungizone ml⁻¹) at 37 °C for up to 10 days. Subsequently, the epidermis was mechanically removed using forceps, and de-epidermalized dermis was cut into 2 × 2 cm squares and placed in culture plates with the papillary dermal surface on the underside. Stainless steel rings were placed on top of the dermis, and human primary dermal fibroblasts (5 × 10⁶ cells) in RM+ medium were inoculated into the rings on the reticular dermal surface. Following overnight incubation, the de-epidermalized dermis was inverted for orientation of the papillary dermal surface on top before the rings were replaced. Retroviral-infected PHAKs (3.0 × 10⁵ cells) in RM+ medium were seeded inside the rings onto the dermis. After 2 days, the dermis was raised to the air–liquid interface in the same orientation by placing the composites on stainless steel grids, and the RM+ medium was refreshed every 3 days. After 10 days, the skin cultures were fixed in 10% buffered formalin and embedded in paraffin.

**Human SCC tissue.** Archival paraffin blocks were used for immunohistochemistry. Ethical approval for this study was obtained from the Ethics Committee at the University of Cologne. Embedded biopsy material was dissected from paraffin sections with sterile disposable scalpels.

**β-HPV genotyping of human tissue.** Tissues were cut and DNA isolation was carried out using a QIAamp DNA mini kit (Qiagen) according to the manufacturer’s instructions. β-HPV genotyping was performed using a broad-spectrum PCR (PM-PCR) reverse hybridization assay (RHA) method (skin β-HPV prototype research assay; Diassay BV) (de Koning et al., 2006). The PM-PCR was specific for amplification of members of the genus *Betapapillomavirus* and targeted a fragment of 117 bp from the E1 region of the HPV genome. Combined with the RHA, it was possible to identify 25 β-HPV types (i.e. HPV-5, -8, -9, -12, -14, -15, -17, -19 to -25, -36 to -38, -49, -73, -75, -76, -80, -92, -93 and -96). The RHA was performed according to the manufacturer’s instructions. As a positive PCR control, a β-PV plasmid clone, was included at an amount approximately 100 times the limit of detection of the assay. The positive control was detected in all cases. Amplification of a 268 bp fragment from the β-globin gene with primers PC04 and GH20 was used to confirm the quality of the isolated DNA samples (Saiki et al., 1988).

**Western blot analysis.** For Western blot analysis, cells were trypsinized, pelleted by centrifugation and lysed on ice for 3 min in RIPA buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS] supplemented with 1× Cocktail Protease Inhibitors (Roche Diagnostics). The resulting extracts were sonicated, and protein concentration was determined using a Bio-Rad Protein Assay. Cell extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After the membrane had been blocked with 5% skimmed milk or 5% BSA in TBST [10 mM Tris/HCl (pH 8.0) 150 mM NaCl, 0.05% Tween 20] for 1 h, the blots were probed with antibodies against lipocalin-2 (goat anti-human lipocalin-2; R&D Systems), involucrin (clone SY5; Sigma) and glycolaldehyde 3-phosphate dehydrogenase (GAPDH) (mouse monoclonal; Abcam), which was used as loading control. Immunoreactive proteins were visualized using HRP-coupled secondary antibodies and a chemiluminescence system (ECL Plus Western Blotting Detection System; GE Healthcare). The blot was exposed to autoradiographic film. To quantify the Western blot bands, we used Quantity One software (Bio-Rad). The lipocalin-2-specific signal was corrected for the GAPDH loading control. The value for the pLXSN condition was set as 1 and other conditions were calibrated correspondingly to allow ratio comparisons.

**ELISA.** To quantify the amount of secreted lipocalin-2, the cell-free culture supernatants of HPV E7-expressing cells were collected, cleared from cellular debris by centrifugation at 1000 r.p.m. for 5 min in a microfuge and stored at −80 °C until use. The cytokine assays were performed using an ELISA according to the manufacturer’s protocols (R&D Systems). Secreted lipocalin-2 amounts were normalized to the number of cells at the time of sample collection and to the total amount of secreted protein (Total Protein Assay; Bio-Rad). Results were expressed as the mean ± SD of three independent experiments.
Immunohistochemistry. Tissue sections (4 μm) of paraffin-embedded organotypic skin cultures or 6 μm sections of human cutaneous skin tumour specimens were de-paraffinized by xylol and ethanol incubations, blocked for 1 h with 20% heat-inactivated FCS and incubated overnight with primary antibodies for lipocalin-2 and filaggrin in 2% FCS. The primary antibodies were omitted in the negative controls. After extensive washing, the primary bound antibodies were detected using Alexa Fluor 594-conjugated donkey anti-goat and Alexa Fluor 488-conjugated goat anti-rabbit (diluted 1:800 in PBS with 2% FCS/Tween 20) antibodies for 1 h at room temperature. Nuclei were counterstained with DAPI (1 μg ml⁻¹; Roche). Sections were mounted with Immumount (Thermo Scientific) and specific signals were visualized by immunofluorescence microscopy.

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