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SHORT COMMUNICATION

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Connexin 43 expression is downregulated in raft cultures of human keratinocytes expressing the human papillomavirus type 16 E5 protein

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Abstract A decrease in gap junction-mediated cell-to-cell communication has previously been observed in monolayer cultures of human keratinocytes (HaCaT cells) expressing the human papillomavirus type 16 E5 (HPV16 E5) gene and attributed to the reduced phosphorylation of connexin 43, the most abundant connexin in HaCaT cells. In line with this observation, we have now analyzed the effect of HPV16 E5 on connexin 43 expression in raft cultures produced by transfected HaCaT cells. These keratinocytes transcribe HPV16 E5 under the control of a dexamethasone-inducible promoter. Our results show that treatment with dexamethasone leads to an almost complete disappearance of connexin 43 in rafts expressing the E5 gene but not in control rafts. In our study we discuss the possible effects of this downregulation on cell-cell communication and cellular malignant transformation.

Key words Raft cultures · Keratinocytes · Gap junctions · Transcription · Papillomavirus · Human

Introduction

Human papillomavirus type 16 (HPV16) sequences have been detected in more than 80% of human cervical tumors (Zur Hausen 1991). Two virus-encoded proteins, E6 and E7, are considered to be oncogenic and capable of transforming keratinocytes and fibroblasts in vitro, while recently another virus-encoded protein, E5, has been found to transform human fibroblasts and increase E7-mediated malignancy (Leechanachai et al. 1992; Bouvard et al. 1994). E5 is a very hydrophobic, small polypeptide of 83 amino acids, located mainly in the Golgi apparatus, endosomes, endoplasmic reticulum, and plasma membrane (Conrad et al. 1993). In monolayer cultures of HaCaT cells, a spontaneously immortalized human keratinocyte cell line, expression of HPV16 E5 correlates with impaired gap junction-mediated cell-to-cell communication (Oelze et al. 1995). This impairment is probably due to E5-mediated dephosphorylation of connexin 43 (Cx43), the most abundant connexin in HaCaT cells (Fitzgerald et al. 1994; Wenzel et al. 1998).

Since the target cell of HPV16 is the cervical epithelium, it would be of interest to analyze the effect of E5 on Cx43 in the context of a stratified epithelium, because results obtained from monolayer cultures cannot be applied to the stratified structure.

In human skin, Cx43 is mostly expressed in the stratum spinosum, whereas the most differentiated layers almost lack this protein (Salomon et al. 1994; Lucke et al. 1999). Interestingly, expression of Cx43 has been found to be reduced in carcinomas derived from stratified epithelia. However, no difference nor even a slight increase was found in papillomas derived from the same tissues (Sawey et al. 1996). Therefore, it appears that expression of Cx43 and malignant transformation are frequently inversely correlated. Indeed, transfection of Cx43 into malignant cells results in reversion of the malignant phenotype, suggesting that Cx43 bears properties of a tumor suppressor (Hirschi et al. 1996).

In this study we have analyzed the behaviour of Cx43 in raft cultures generated with HaCaT cells expressing the HPV16 E5 gene. Raft cultures of HaCaT cells reconstitute an epithelial histoarchitecture in vitro and exhibit, to a large extent, the proliferation and differentiation patterns of a stratified squamous epithelium (Boelsma et al. 1999; Schoop et al. 1999). In this communication we demonstrate that expression of HPV16 E5 in raft cultures correlates with a strong downregulation of Cx43.

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Materials and methods

Establishment of raft cultures

Raft cultures were produced with HPV16 E5- and empty vector (pMSG)-transfected HaCaT cells under standard conditions (Schoop et al. 1999). Briefly, cell-containing collagen lattices were established by suspending 1.5×10^5 fibroblasts in FCS, followed by addition of the suspension to 1 ml ice-cooled collagen solution (4 mg/ml; IMB, Germany). After polymerization at 37°C for 25 min, 5×10^5 E5 or pMSG cells were seeded on the surface of each collagen matrix. Dexamethasone was added to a concentration of 1 µM to induce transcription of the E5 gene (Oelze et al. 1995).

In situ hybridization

In situ hybridization (ISH) was carried out using the protocol as described (Bosch et al. 1993). Briefly, frozen sections were fixed in paraformaldehyde and then digested with proteinase K. After overnight hybridization with a ^{32}P-labeled E5-specific riboprobe at 45°C, in the presence of 50% formamide in 2×SSC, slides were extensively washed and treated with RNase to remove unbound probe from the tissues. After washing, slides were dipped into NTB2 emulsion (Kodak) and exposed and counterstained with hematoxylin and eosin (HE).

Indirect immunofluorescence

To analyze Cx43 localization, cryostat sections (8–10 µm) were mounted on adhesive slides, air dried, and fixed in cold methanol-acetone before incubation of the tissues with the mouse monoclonal antibody against Cx43 (Transduction Laboratories) in a humid chamber overnight at 4°C. After washing, the secondary antibody labeled with Cy-3 was added for 1 h at room temperature. Samples were embedded in Vectashield and photographed using a Zeiss inverted microscope (Zeiss ICM-405).

Results

Morphological features of raft cultures

HaCaT cells have been widely used to generate raft cultures in vitro (Boelsma et al. 1999; Schoop et al. 1999). In our study, HaCaT cells, stably transfected with the HPV16 E5 gene or the empty vector (see Oelze et al. 1995), formed a stratified epithelium with up to six layers and flattening of the cells in the uppermost layers, resembling normal skin epithelium. The differentiation characteristics of our raft cultures were assessed by indirect immunofluorescence with an antibody to cytokeratins 1 and10 (CKs1.10), which act as markers for early epidermal differentiation. As shown in Fig. 1a,b, immunostaining of 7-day-old cultures with the CKs1.10 antibody indicated a clear cytokeratin expression. Most of the staining was observed in the suprabasal layers of the raft, comparable with the pattern of early epidermal differentiation. Furthermore, fluorescence intensity and distribution of CKs1.10 was similar in both E5 and control rafts, indicating that expression of the viral gene did not cause an alteration of structural features and early differentiation.

Detection of E5 mRNA in raft cultures by in situ hybridization

Since no antibody to E5 is available as yet, E5 gene expression was analyzed in the raft cultures treated with dexamethasone by in situ hybridization to determine the presence and distribution of specific RNA. Brightfield (Fig. 1c) and darkfield (Fig. 1d) micrographs of E5 rafts revealed that E5 mRNA was homogeneously distributed within the entire epithelium. In contrast, no hybridization was detected with a radiolabeled sense probe (Fig. 1e). A weak E5 gene expression was observed in the absence of dexamethasone (Fig. 1f) owing to the fact that the promoter used is slightly leaky (Oelze et al. 1995). Furthermore, no hybridization was detected when control raft cultures were used (results not shown), demonstrating that the observed signal was specific for E5 transcripts and transcription was restricted to HPV16 E5-transfected keratinocytes.

Expression of Cx43 under basal conditions

To investigate whether E5-transfected HaCaT keratinocytes modulate expression and spatial distribution of Cx43 in raft cultures, we first analyzed Cx43 expression without induction of E5 expression by dexamethasone. One-week-old raft cultures were fixed and immunostained with the antibody to Cx43. As shown in Fig. 1g and h, a similar amount and distribution of Cx43 was observed up to the suprabasal cell layers in both E5-expressing and control cultures, with a clear, grain-like distribution at the cell membranes (Fig. 1i,j). This expression pattern remained constant after 2 weeks and 3 weeks in culture, with only a slight reduction in the amount of Cx43.

Effect of E5 expression on Cx43

In a further step, we analyzed the effect of E5 expression on the presence and distribution of Cx43. Raft cultures were treated with dexamethasone, to induce transcription of the E5 gene, and indirect immunofluorescence performed as described before. Surprisingly, a drastic decrease in Cx43 expression in E5-expressing rafts was observed after 1 week in culture, with only remnants at the plasma membrane of the basalmost keratinocytes (Fig. 1k,m). In contrast, rafts generated with control-transfected keratinocytes exhibited a clear Cx43 immunolocalization at the cell surface as well as intracellularly in basal and parabasal layers (Fig. 1l,n). Cx43 was not observed in the uppermost layers, reminiscent of the expression pattern found in normal human skin, where the most differentiated layers are devoid of Cx43 (Salomon et al. 1994; Lucke et al. 1999).

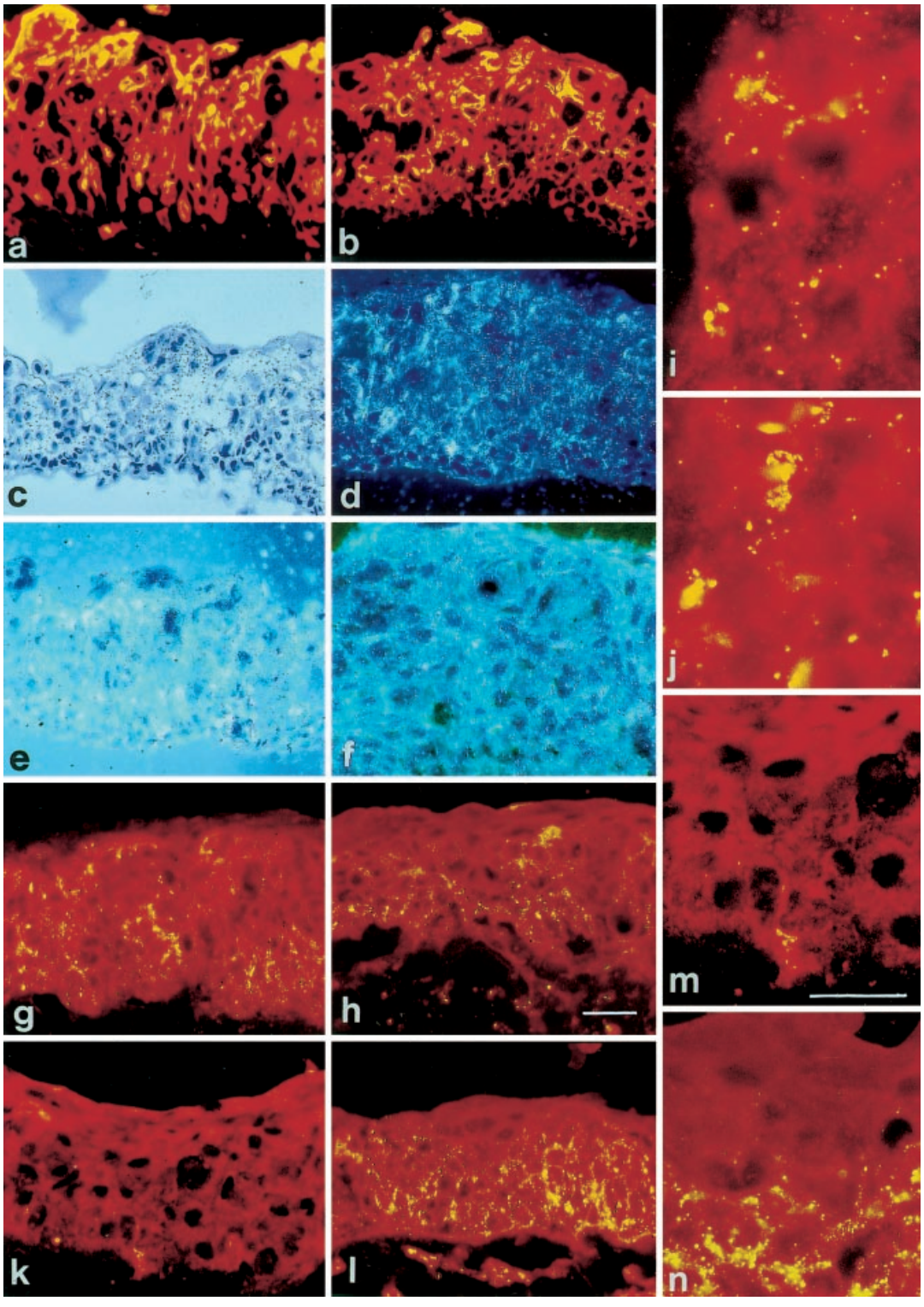

Fig. 1a–n Suprabasal expression of CKs1.10 in 7-day-old raft cultures of E5-transfected (**a**) and control-transfected (**b**) HaCaT cells detected by indirect immunofluorescence (IIF). In situ hybridization of an E5-specific riboprobe with E5-expressing HaCaT cells treated with dexamethasone (**c**, brightfield; **d**, darkfield) reveals homogeneous distribution of E5 transcripts within the epithelium. The specificity of the reaction is proved by the lack of hybridization signals, as shown in **e** using an E5 sense riboprobe. In **f,** hybridization was performed with rafts untreated with dexamethasone. Only few hybridization signals are visible. Expression of Cx43 in rafts of E5-transfected (**g**) and control-transfected (**h**) cells in the absence of dexamethasone. A comparable decoration, preferentially localized in the suprabasal layers, is observed. Most of the IIF signals are localized at the plasma membrane, as shown in higher magnifications for E5 rafts (**i**) and control rafts (**j**). Treatment with dexamethasone leads to an almost complete disappearance of Cx43 in E5-expressing rafts (**k**) but not in controls (**l**). Detailed views of **k** and **l** are given in **m** (E5) and **n** (control cells). *Bars* **a–h**,**k,l** 35 µm; **i,j,m**,**n** 15 µm

Discussion

Expression of the HPV16 E5 protein in monolayer cultures of human keratinocytes results in dephosphorylation of Cx43 and impairment of gap junction-mediated cell-cell communication (Oelze et al. 1995). Nevertheless, whether these results can also be applied to an in vivo multilayered epithelium is rather improbable. For this reason, we used organotypic cultures in vitro, which fairly reproduce the normal structure of a stratified epithelium. In this study, we describe the effect of HPV16 E5 on the expression and distribution pattern of Cx43 in raft cultures of human keratinocytes.

A similar raft model to analyze the effects of HPV31 E5 expression on the EGF receptor family has already been used involving epithelial cells from a cervical intraepithelial neoplasia (Mayer and Meyers 1998). However, the applicability of this model was limited, since only small quantities of E5 were expressed by these cells, thus restricting the conclusions concerning the effects of the viral gene (Mayer and Meyers 1998).

Hence, we modified the approach by generating raft cultures where all cells transcribe the E5 gene from an inducible promoter. Our in situ hybridizations revealed that E5-specific mRNA is observed in similar amounts in all epithelial layers of the raft cultures. This expression did not affect cellular proliferation, since no differences were found in the amount of Ki-67-positive cells between E5-expressing and control rafts (unpublished results). In addition, the comparable CK1.10 expression patterns found in E5 and control cells suggest that the viral protein did not change the early differentiation properties of these keratinocytes.

Our data demonstrate that the expression and distribution of Cx43 was similar in E5-expressing and control raft cultures, when the E5 gene is noninduced, while this pattern changes drastically after addition of dexamethasone to induce E5 transcription. Whereas in control cultures a high expression of Cx43 was found in the basal and parabasal epithelial layers of the raft culture, almost all connexin had already disappeared in the E5-expressing cultures 1 week after induction.

Since in noninduced raft cultures the amount and distribution of Cx43 was similar in E5 cultures and control cultures, this downregulation of Cx43 is correlated with and therefore may be a consequence of E5 expression. As dexamethasone was added to both E5 and control cultures, it is highly unlikely that it elicits two different reactions in the two cultures. Furthermore, no experimental evidence for a direct effect of dexamethasone on Cx43 expression in stratified epithelia has been reported. In liver cells, however, dexamethasone has been demonstrated to modulate the expression of Cx32 and Cx26, but this is not the case for Cx43 (Ren et al. 1994). In our experiments, in contrast to E5-expressing rafts, dexamethasone leads to a slight increase in Cx43 signal intensity in 7-day-old control rafts.

Although the reasons for the differences observed in the E5-mediated Cx43 modulation between monolayers (Oelze et al. 1995) and raft cultures are unknown, it can be hypothesized that they are based on the epithelial histoarchitecture of the raft culture. However, the exact mechanisms remain unclear.

It has been reported frequently that malignancy correlates with a decrease in Cx43 expression in several tissues (Fitzgerald et al. 1994; Budunova et al. 1995; Cesen-Cummings et al. 1998). The effect of this decrease is probably a reduced cell-cell communication impairing homeostatic growth control by the surrounding cells. We have already hypothesized that E5 renders HPV-infected cells more susceptible to growth factors and, through the loss of gap junctions, more insensitive to control signals from surrounding, noninfected cells (Oelze et al. 1995). The data presented in this paper support this hypothesis and suggest that loss of cell-cell communication is probably one of the first steps towards malignancy.

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