No evidence for induction of key components of the Notch signaling pathway (Notch-1, Jagged-1) by treatment with UV-B, 1,25(OH)_{2}D_{3}, and/or epigenetic drugs (TSA, 5-Aza) in human keratinocytes in vitro

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Abbreviations: 5-Aza, 5-azacytidine; BCC, basal cell carcinoma; Dll, Delta-like; DNMT1, DNA methyltransferase inhibitor; HDACI, histone deacetylase inhibitor; Hes, hairy and enhancer of split; Hrt, Hes-related transcription factor; Jag, Jagged; MAML, mastermind-like; miRNA, micro ribonucleic acid; NICD, Notch intracellular domain; SCC, squamous cell carcinoma; TSA, trichostatin A; VDR, vitamin D receptor

Notch signaling is of high importance for growth and survival of various cell types. We now analyzed the protein expression of two key components of the Notch signaling pathway (Notch-1, Jagged-1) in spontaneously immortalized (HaCaT) and in malignant (SCL-1) human keratinocytes, using western analysis. We found that Notch-1 and its corresponding ligand Jagged-1 are expressed in both cell lines, with no marked change following UV-B treatment. Moreover, treatment of both cell lines before or after UV-B irradiation with 1,25-dihydroxyvitamin D_{3}, the biologically active form of vitamin D, and/or epigenetic modulating drugs (TSA; 5-Aza) did not result in a marked modulation of the protein expression of Notch-1 or Jagged-1. Under the experimental conditions of this study, treatment with 1,25(OH)_{2}D_{3} protected human keratinocytes in part against the antiproliferative effects of UV-B-radiation. In conclusion, our findings do not point at a differential expression of these two key components of Notch signaling in non-malignant as compared to malignant human keratinocytes, indicating that alterations in their expression are not of importance for the photocarcinogenesis of human squamous cell carcinomas. Moreover, our findings do not support the hypothesis that modulation of Notch signaling may be involved in the photoprotective effect of 1,25-dihydroxyvitamin D_{3}, that we and others reported previously. Additionally, we demonstrate that epigenetic modulating drugs (TSA; 5-Aza) do not markedly modulate the expression Notch-1 or Jagged-1 in UV-B-treated human keratinocytes in vitro.

Introduction

UV-radiation is the most important environmental risk factor for non-melanoma skin cancer, causing photocarcinogenesis of basal cell carcinomas (BCC) and cutaneous squamous cell carcinomas (SCC). These types of skin cancer are the most common malignancies in Caucasian populations with annual incidence rates in the United States of nearly 1 million and 250,000 cases, respectively. In contrast to BCCs that in general do not metastasize, SCCs are characterized by biologically more aggressive growth and exert metastatic potential with frequencies approaching 12.5%. In many cases, they develop from characteristic precursor lesions (actinic keratoses), that are nowadays accepted to represent in situ carcinomas. Mutations in genes involved in the sonic hedgehog (SHH) signaling pathway have been shown to be of high importance for the carcinogenesis of both human and mouse BCC. Until today, no gene mutation that uniquely underlies human SCCs is known, although UV-induced mutations in the p53 gene are a key event in the photocarcinogenesis of SCCs. We and others have shown previously that the biologically active vitamin D metabolite 1,25-dihydroxyvitamin D \([1,25(\text{OH})_{2}\text{D}]\) and its analogs have the capacity to protect cultured human keratinocytes in part against the hazardous effects of UV-radiation.

It was the aim of this study to gain more insights into the role of vitamin D- and Notch-signaling pathways for the photocarcinogenesis of SCC, using cultured non-malignant (HaCaT) and malignant (SCL-1) keratinocytes as an in vitro model to investigate potential characteristic changes during the process of...
malignant transformation of human keratinocytes. Understanding the role of Notch signaling for the photocarcinogenesis of SCC is of high relevance, given the enormous impact of Notch signaling for cellular processes that are implicated in carcinogenesis, including cellular proliferation/differentiation. Decades ago, the Notch signaling pathway was first described as the key regulator of ectodermal specification and neurogenesis in Drosophila. More recently, it has been shown that Notch signaling is of critical importance not only for the embryonic development but also for the growth of various cell types, including human keratinocytes. It has now convincingly been demonstrated that the Notch pathway regulates a broad variety of cellular processes, including differentiation, proliferation, angiogenesis, apoptosis and cell fate decisions, that are of high importance for organogenesis and tissue homeostasis that are involved in skin carcinogenesis. Underlining its importance, Notch signaling is highly conserved during evolution, developmentally regulated, and cell type dependent. In general, Notch signaling regulates developmental processes through binary decision, lateral inhibition and boundary formation. Notch-mediated cell-cell communication is mediated via the coordinated and differential expression of Notch receptors and their corresponding ligands on the cell surface. At present, four evolutionary conserved transmembrane Notch receptors (Notch1–4) are known in mammals, that are activated by five corresponding Notch ligands of the Delta (Delta-like 1, 3 and 4) and Jagged (Jagged1 and 2) families. Ligand-induced Notch receptor activation causes cleavage of the Notch intracellular domain (NICD). The NICD then translocates to the nucleus and forms a ternary complex with the transcriptional coactivator, mastermind-like (MAML) protein, as well as the DNA-binding protein, CSL, which regulates DNA-biding specificity and target gene expression. Notch-mediated cell-cell communication is mediated via the coordinated and differential expression of Notch receptors and their corresponding ligands on the cell surface. At present, four evolutionary conserved transmembrane Notch receptors (Notch1–4) are known in mammals, that are activated by five corresponding Notch ligands of the Delta (Delta-like 1, 3 and 4) and Jagged (Jagged1 and 2) families. Ligand-induced Notch receptor activation causes cleavage of the Notch intracellular domain (NICD). The NICD then translocates to the nucleus and forms a ternary complex with the transcriptional coactivator, mastermind-like (MAML) protein, as well as the DNA-binding protein, CSL, which regulates DNA-biding specificity and target gene expression.

In healthy and diseased human skin, Notch receptors and their ligands are differentially expressed and localized in the different cell layers of the viable epidermis. However, many of their specific functions are until today not completely understood. In healthy “normal” skin Notch1 and the corresponding ligands Delta1 and Jagged1 are expressed in all viable cell layers of the epidermis, with the strongest detection of Delta1 and Jagged 1 in keratinocytes of the epidermal basal layer. It was reported that signaling mediated by Delta/Notch is increased when cells undergo a normal and regulated differentiation program, as in keratinocyte layers of the normal adult human epidermis. In contrast, Notch signaling has been reported as reduced in psoriasis vulgaris and other hyperproliferating skin diseases. Moreover, loss of Notch1 in young mice has been shown to cause hyperproliferation of the basal epidermal layer and to change expression of many markers involved in cellular differentiation and proliferation, including p21 (reduced) and Gli2 (elevated). In epidermal epithelial cells, activation of Notch1 induces p21 expression in a CSL-dependent manner, resulting in cell cycle withdrawal and terminal differentiation. Additionally, Notch1 stimulates caspase 3 activity, that promotes terminal differentiation of embryonic keratinocytes. However, the potential physiological/pathophysiological function and the regulation of the Notch pathway in the photocarcinogenesis of SCCs are largely unknown. While it has been demonstrated that other molecular pathways modulate the activity of the Notch pathway, little is known about the impact of UV-B or vitamin D compounds on Notch signaling. We and others have previously reported that 1,25-dihydroxyvitamin D and analogs protect human keratinocytes against the hazardous effects of UV-radiation. Furthermore, increasing evidence now clearly indicates that the vitamin D endocrine system is implicated in pathogenesis and progression of various malignancies, including the photocarcinogenesis of human cutaneous SCC. It is well known that 1,25-dihydroxyvitamin D3, the biologically most active natural vitamin D metabolite, and its analogs, act via binding to a corresponding intranuclear receptor (VDR), present in target tissue cells. In various normal and malignant cell types the effects of 1,25-dihydroxyvitamin D3 include the regulation of cell growth and differentiation. It can be speculated whether pharmacologic regulation of vitamin D- or Notch-signaling may represent a new target for cancer prevention and therapy. Modulation of Notch and/or vitamin D-signaling pathways may represent a potential novel strategy to prevent the photocarcinogenesis of and to treat cutaneous SCCs. It was the aim of this study to gain further insights into the role of Notch- and vitamin D signaling pathways for photocarcinogenesis of SCCs by analyzing the protein expression of two key components of Notch signaling (Notch1, Jagged 1), and their modulation by treatment with UV-B and/or 1,25-dihydroxyvitamin D3, and epigenetic modulating drugs (TSA, 5-Aza) in non-malignant (HaCaT) and in malignant (SCL-1) keratinocyte cell lines in vitro.

Results

Treatment with 1,25(OH)2D3 protects human keratinocytes in part against the antiproliferative effects of UV-B-radiation. As shown in Figure 1 for HaCaT keratinocytes, treatment with 1,25(OH)2D3 protects under our experimental conditions (crystal-violet staining of cells as described in Materials and Methods) human keratinocytes in part against the antiproliferative effects of UV-B-radiation. Please note that 24 h following treatment with UV-B and 1,25(OH)2D3, 1,25(OH)2D3-mediated effects are more pronounced in cells treated with 100 J UV-B as compared with those treated with 500 J UV-B (Fig. 1). Please also note that epigenetic drugs TSA and 5-Aza as well modulate the proliferation of HaCaT keratinocytes treated with and without UV-B (Fig. 1).

Protein expression of key components of the Notch signaling pathway in human keratinocyte cell lines. Western analysis revealed that Notch-1 and its corresponding ligand Jagged-1 are expressed in non-malignant (HaCaT keratinocytes) and in malignant (SCL-1 cells) human keratinocytes. Results of representative experiments in HaCaT keratinocytes are shown in Figures 2 and 3.

Treatment with UV-B and/or 1,25(OH)2D3 does not exert strong effects on protein expression of Notch-1 in non-malignant
and in malignant human keratinocytes in vitro. Western analysis revealed that treatment with UV-B and/or 1,25(OH)_{2}D_{3} does not exert strong effects on protein expression of Notch-1 in non-malignant (spontaneously immortalized HaCaT keratinocytes) or in malignant (cutaneous squamous carcinoma cells SCL-1) human keratinocytes. A representative experiment demonstrating protein expression of Notch-1 in HaCaT keratinocytes 24 h following treatment with UV-B [(A) 0 J; (B) 100 J; (C) 500 J] alone or together with 1,25(OH)_{2}D_{3} (10^{-8} M) is shown in Figure 2. Note that no pronounced visual differences in staining intensity for Notch-1 were seen in protein extracts from controls as compared with those from UV-B- and/or 1,25(OH)_{2}D_{3}-treated cells (Fig. 2). Results in HaCaT keratinocytes were comparable with those in SCL-1 cells (data not shown).

Treatment with UV-B and/or 1,25(OH)_{2}D_{3} does not exert strong effects on protein expression of Jagged-1 in non-malignant or in malignant human keratinocytes. Western analysis revealed that treatment with UV-B and/or 1,25(OH)_{2}D_{3} does not exert strong effects on protein expression of Jagged-1 in non-malignant (spontaneously immortalized HaCaT keratinocytes) or in malignant (cutaneous squamous carcinoma cells SCL-1) human keratinocytes. A representative experiment demonstrating protein expression of Jagged-1 in HaCaT keratinocytes 24 h following treatment with UV-B [(A) 0 J; (B) 100 J; (C) 500 J] alone or together with 1,25(OH)_{2}D_{3} (10^{-8} M) is shown in Figure 3. Note that no strong visual differences in staining intensity for Jagged-1 were seen in protein extracts from controls as compared with those from UV-B- and/or 1,25(OH)_{2}D_{3}-treated cells (Fig. 3). Results in HaCaT keratinocytes were comparable with those in SCL-1 cells (data not shown).

Treatment with epigenetic modulating drugs (TSA, 5-Aza), alone or in combination with UV-B and/or 1,25(OH)_{2}D_{3}, does not exert strong effects on protein expression of Notch-1 in non-malignant or in malignant human keratinocytes. Western analysis revealed that treatment with epigenetic modulating drugs (TSA, 5-Aza) does not exert pronounced effects, alone or in combination with UV-B and/or 1,25(OH)_{2}D_{3}, on protein expression of Notch-1 in non-malignant (spontaneously immortalized HaCaT keratinocytes) or in malignant (cutaneous squamous carcinoma cells SCL-1) human keratinocytes (Fig. 2). A representative experiment demonstrating protein expression of Notch-1 in HaCaT keratinocytes 24 h following treatment with UV-B [(A) 0 J; (B) 100 J; (C) 500 J] alone or together with 1,25(OH)_{2}D_{3} (10^{-8} M) is shown in Figure 2. Note that no strong visual differences in staining intensity were seen in controls as compared with treated cells (Fig. 2). Results in HaCaT keratinocytes were comparable with those in SCL-1 cells (data not shown).

Treatment with epigenetic modulating drugs (TSA, 5-Aza), alone or in combination with UV-B and/or 1,25(OH)_{2}D_{3}, does not exert strong effects on protein expression of Jagged-1 in non-malignant or in malignant human keratinocytes. Western analysis revealed that treatment with epigenetic modulating drugs (TSA, 5-Aza) does not exert strong effects, alone or in combination with UV-B and/or 1,25(OH)_{2}D_{3}, on protein expression of Jagged-1 in non-malignant (spontaneously immortalized HaCaT keratinocytes) or in malignant (cutaneous squamous carcinoma cells SCL-1) human keratinocytes (Fig. 3). A representative experiment demonstrating protein expression of Jagged-1 in HaCaT keratinocytes 24 h following treatment with UV-B [(A)
Discussion

The hazardous effects of solar or artificial UV (UV) radiation, most importantly of UV-B with a wavelength range between 290 and 320 nm, represent an important etiological factor in the development of non-melanoma skin cancer.2-4 UV-B induces photochemical alterations in the skin that may lead to acute effects including sunburn and immune suppression or to chronic effects such as premature skin aging and non-melanoma skin cancer.2-4 UV-B-mediated biological effects in target cells include the induction of apoptosis12,13 and the production of interleukin-6 (IL-6).11 Apoptosis, as a mode of programmed cell death, is induced following UV-B-irradiation when cellular damage is too severe to be repaired.12,13 To induce apoptosis, UV-B modulates a variety of important cellular signaling pathways that involve various nuclear and cell surface death receptors and the formation of reactive oxygen species (ROS).11 Also involved in this process is the activation of a cascade of cystein proteases called caspases.11 The final effector protease, caspase 3, causes cleavage of several substrates, including PARP, which immediately results in apoptosis.11 This cascade appears to be crucial for executing apoptosis induced by UV-B.11 Furthermore, it has recently been described that C-Jun-NH₂-terminal kinase (JNK), a member of the mitogen-activated protein kinases (MAPK), is required for UV-induced apoptosis via the induction of cytochrome c release.11 It has been hypothesized that JNK-dependent apoptosis is mediated through mitochondrial cytochrome c release, which has also been observed as an early event in UV-mediated apoptosis in HaCaT cells.11 On the other hand, UV-B-irradiation strongly induces accumulation of IL-6 mRNA and release of IL-6 protein by human keratinocytes.11 The cytokine IL-6 is an important mediator of the sunburn reaction and of UV-B-dependent immune suppression.12 Furthermore, IL-6 has been implicated in the tumorigenesis of basal cell carcinoma, a frequent neoplasm that can be induced by UV-B radiation.12,13

Our results reported here, demonstrating that 1,25(OH)₂D₃ protects cultured HaCaT keratinocytes and SCL-1 cells in part against the antiproliferative effects of UV-B-radiation, are in line with findings published previously by us and other investigators. We
and others have previously shown that the biologically active vitamin D metabolite 1,25(OH)\(_2\)D and its analogs protect human skin cells from UV-induced hazardous effects, including cell death and apoptosis.\(^{11-13}\) However, it is not completely understood, to what extend effects of 1,25(OH)\(_2\)D on apoptosis, IL-6, PARP, JNK or MAPK are responsible for this protective effect. In previous studies, cytoprotective effects of 1,25(OH)\(_2\)D on UV-B-irradiated keratinocytes were seen morphologically and using a colorimetric cell survival assay.\(^{11}\) Moreover, using an ELISA that detects DNA-fragmentation, it was shown that pretreatment with 1,25(OH)\(_2\)D suppressed UV-B-induced apoptotic cell death.\(^{11}\) Pretreatment of keratinocytes with 1,25(OH)\(_2\)D for 24 h reduced UV-B-stimulated apoptosis by 55–70%. It has been shown that this reduction required relatively high, pharmacological concentrations of 1,25(OH)\(_2\)D and a preincubation period of several hours.\(^{11}\) Moreover, it has been reported that pretreatment with 1,25(OH)\(_2\)D also inhibits mitochondrial cytochrome c release (90%), a hallmark event of UV-B-induced apoptosis.\(^{11}\) Additionally, it has been shown that 1,25(OH)\(_2\)D affects two important mediators of the UV-response: it reduces c-Jun-NH\(_2\)-terminal kinase (JNK) activation and interleukin-6 (IL-6) production.\(^{11}\) Analyzing the cleavage of poly (ADP-ribose) polymerase (PARP) further corroborated these observations. As mentioned before, PARP-cleavage is clearly induced by UV-B-irradiation. In recent studies, it has been shown that pretreatment of keratinocytes with 1,25(OH)\(_2\)D (1 µM for 24h) efficiently, but not completely, inhibited this UV-B-induced PARP-cleavage.\(^{11}\) Apart from these effects, MT-induction may be relevant for the anti-UV-B effects of 1,25(OH)\(_2\)D. MT acts as a radical scavenger in oxygen-mediated UV-B-injury.\(^{11}\) MTs are a class of small cysteine-rich proteins that bind and exchange heavy metal ions but also have clear scavenging properties for ROS.\(^{11}\) Indeed, part of the UV-B-induced damage to cells occurs through the formation of ROS and antioxidative agents such as MT have been reported to be photoprotective.\(^{11}\) MT mRNA expression was clearly induced by 1,25(OH)\(_2\)D. Following these investigations, the anti-apoptotic effect of 1,25(OH)\(_2\)D in keratinocytes was confirmed, using cisplatin and doxorubicin as apoptotic triggers.\(^{13}\) In that study, it was demonstrated that 1,25(OH)\(_2\)D activated two independent survival pathways in keratinocytes: the MEK/extracellular signal regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway.\(^{13}\) Activation of ERK and Akt by 1,25(OH)\(_2\)D was transient, required a minimal dose of 10\(^{-9}\) M and could be blocked by actinomycin D and cycloheximide. Moreover, inhibition of Akt or ERK activity with respectively a PI-3K inhibitor (LY294002) or MEK inhibitors (PD98059, UO126), partially or totally suppressed the anti-apoptotic capacity of 1,25(OH)\(_2\)D.

Finally, 1,25(OH)\(_2\)D changed the expression of different apoptosis regulators belonging to the Bcl-2 family. 1,25(OH)\(_2\)D treatment increased levels of the anti-apoptotic protein Bcl-2 and decreased levels of the pro-apoptotic proteins Bax and Bcl-xL.
levels of the pro-apoptotic proteins Bax and Bad in a time- and dose-dependent way.\textsuperscript{13} The authors concluded that 1,25(OH)\textsubscript{2}D protects keratinocytes against apoptosis by activating the MEK/ERK and the PI-3K/Akt survival pathways and by increasing the Bcl-2 to Bax and Bad ratio. Taken together, these findings suggested the existence of a photoprotective effect of active vitamin D and created new perspectives for the pharmacological use of active vitamin D compounds in the prevention of UV-B-induced skin damage and carcinogenesis.\textsuperscript{11-14} It is well known that photocarcinogenesis of skin cancer is caused largely by mutations at sites of incorrectly repaired DNA photoproducts, of which the most common are the cyclobutane pyrimidine dimers (CPDs).\textsuperscript{40,41} Recently, it has been demonstrated that 1,25(OH)\textsubscript{2}D protects primary human keratinocytes against the induction of CPDs by UVB.\textsuperscript{14} This protection required pharmacologic doses of 1,25(OH)\textsubscript{2}D and an incubation period of at least 8 h before irradiation. It has been speculated that the anti-proliferative capacity of 1,25(OH)\textsubscript{2}D underlies its protective effect against UVB-induced DNA damage.\textsuperscript{14} De Haes et al. demonstrated that 19-nor-14-epi-23-yne-1,25(OH)\textsubscript{2}D (TX 522) and 19-nor-14,20-bisepi-23-yne-1,25(OH)\textsubscript{2}D (TX 527), two low-calcemic analogs of 1,25(OH)\textsubscript{2}D, were even 100 times more potent than the parent molecule in inhibiting UV-B-caused DNA damage.\textsuperscript{14} It was speculated that these molecules therefore may represent promising candidates for the chemoprevention of UV-B-induced skin cancer.\textsuperscript{14} Other investigators showed that treatment with three different vitamin D compounds [1,25(OH)\textsubscript{2}D]; the rapid acting, low calcemic analog, 1\alpha,25(OH)(2)lumisterol(3) (JN); and the low calcemic but transcriptionally active hybrid analog 1\alpha-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitamin D\textsubscript{3} QW-1624F\textsubscript{2}–2 (QW)] diminished in all skin cell types the numbers of UV-induced pre-mutagenic CPDs from 0.5 h after cessation of UV radiation, which may explain the enhanced cell survival of skin cells.\textsuperscript{42} In that study, the rapid response antagonist analog 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} (HL) abolished the photoprotective effects of 1,25(OH)\textsubscript{2}D while a genomic antagonist, (23S)-25-dehydro-1\alpha-hydroxyvitamin D\textsubscript{3} lactone (TEI-9647), had no effect.\textsuperscript{42} UV radiation increased p53 expression in human skin cells, while concurrent treatment with 1,25(OH)\textsubscript{2}D further enhanced this effect several fold, at 3 and 6 h after UV radiation.\textsuperscript{42} Combined with previously reported lower nitrite levels with 1,25(OH)\textsubscript{2}D, it has been speculated that this increased p53 expression may favor DNA repair over apoptosis.\textsuperscript{42} Additionally, it has convincingly been shown that topical application of 1,25(OH)\textsubscript{2}D or QW suppressed solar simulated UV (SSUVR)-induced pyrimidine dimers in the epidermis of irradiated hairless Shh:HR1 mice, measured 24 h after irradiation.\textsuperscript{42} Furthermore, UV-induced immunosuppression in the mice was markedly reduced by topical application of either 1,25(OH)\textsubscript{2}D or QW.\textsuperscript{42} Taken these data together, they convincingly show a protective effect of vitamin D compounds against UV-B-induced photodamage in vitro and in vivo. It is tempting to speculate that the UV-B-induced cutaneous production of vitamin D may represent an evolutionary highly conserved feedback mechanism that protects the skin from the hazardous effects of solar UV-radiation.

Our results reported here, demonstrating that 1,25(OH)\textsubscript{2}D protects cultured HaCaT keratinocytes and SCL-1 cells in part against the antiproliferative effects of UV-B-radiation, are in line with the findings published previously that are outlined above.

Next, we analyzed whether co-treatment with epigenetic drugs 5-Aza or TSA modulates biologic effects of UV-B and/or 1,25(OH)\textsubscript{2}D in cultured human HaCaT keratinocytes or in SCL-1 cells. Interestingly, both compounds alone exerted antiproliferative effects in both cell lines. Antiproliferative effects of combined treatment with 500 J UV-B and 5-Aza or TSA were stronger as compared with treatment with 500 J UV-B, 5-Aza or TSA alone. In most cases, antiproliferative effects of combined treatment with UV-B, 1,25(OH)\textsubscript{2}D and 5-Aza or TSA were slightly smaller as compared with treatment with UV-B and 5-Aza or TSA alone.

Next, we analyzed whether protein expression of two key components of the Notch signaling pathway, namely Notch-1 and Jagged-1, is modulated by treatment with UV-B and/or 1,25(OH)\textsubscript{2}D in HaCaT and/or SCL-1 cells. It has been reported previously that Notch-1 deletion in epidermal keratinocytes causes skin carcinogenesis, while in contrast Notch-1 acts in most other tissues as a proto-oncogene.\textsuperscript{5,43} Recently, the mechanisms underlying the carcinogenesis-promoting properties of Notch-1-deficient skin have been analyzed in mice characterized by a chimeric or global deletion pattern in their epidermis.\textsuperscript{5,44} Findings of this investigation obtained by deleting Notch-1 either before or after DMBA treatment in the K14CreERT system indicate that loss of Notch-1 is not of importance for the initiating event of multistage skin carcinogenesis.\textsuperscript{5,45} However, it was demonstrated that Notch-1 loss acts as a skin cancer-promoting event. In this investigation, delaying Notch-1 deletion in K14CreERT mice until after the tumor-promotion stage of carcinogenesis demonstrated that late deletion of Notch-1 contributed to malignant progression of benign papillomas, a phenotype that is found upon loss of p53 but not loss of p21\textsuperscript{WAF1/Cip1},\textsuperscript{5,46} a specific Notch-1 target in the skin.\textsuperscript{5,22} In summary, the authors of this study concluded that the main effect of Notch-1 loss in skin carcinogenesis is to support the initiated cells with a proliferative signal to promote tumor growth and proceed to invasive skin cancer. It has been speculated that this proliferative signal is located downstream of Notch-1 loss and could be delivered from within the initiated cells, supporting Notch-1’s role as a classical tumor suppressor in epidermal keratinocytes.\textsuperscript{5,26}

As an alternative pathway, it has been hypothesized that this signal could be sent by the skin microenvironment reacting to Notch-1 loss in the epidermis.\textsuperscript{5,46-50} The experimental system used by Demehri et al.\textsuperscript{5,44} was designed to distinguish between these two possibilities. In their investigation, the chimeric pattern of Notch-1 deletion by Mxs2-Cre resulted in neighboring territories of Notch-1-expressing and Notch-1-deficient keratinocytes coexisting in the same microenvironment.\textsuperscript{5,44} Investigating a large number of tumors isolated from DMBA/TPA-treated Mxs2-NICKO mice clearly showed that tumors comprised mostly (\~99\%) of Notch-1-expressing cells were as likely to form as tumors comprised predominantly of Notch-1-deleted cells in the same environment.\textsuperscript{5,44} The authors concluded that
epidermal Notch-1 loss generates a non-cell autonomous signal, promoting tumorigenesis from any initiated cell exposed to the microenvironment conditioned by Notch-1-deficient keratinocytes.5,44 These results underline the relevance of the microenvironment as an active contributor to tumor development5,51 by demonstrating that it can be the primary source of proliferative signals to initiated cells.

In summary, these investigations impressively demonstrate the importance of the Notch signaling pathway for the growth characteristics of non-malignant and malignant human keratinocytes. Interestingly, in vitro treatment of HaCaT and SCL-1 cells with UV-B, 1,25(OH)2D, TSA or 5-Aza, did not result in a modulation of the protein expression of Notch-1 and Jagged 1, two key components of the Notch signaling pathway. In conclusion, our findings do not point at a differential regulation of the protein expression of Notch-1 and Jagged 1 in non-malignant or in malignant keratinocytes by treatment with UV-B, 1,25(OH)2D, TSA or 5-Aza. It has been speculated that pharmacologic modulation of Notch signaling could be a promising new target for the treatment of skin cancer.52 It was shown that the topical treatment with the immunomodulatory agent imiquimod selectively upregulates gene expression of Notch-1 and corresponding ligands (Jagged 1, Delta 1) in BCC.52 Imiquimod represents a strong immune response modifier that mediates its effects via activation of toll-like receptor 7 (TLR 7), thereby resulting in an activation of NF-κB,52 elevated synthesis of proinflammatory cytokines and a potent increase of anti-tumor Th1 immunity.52 Interestingly, Jagged 1 is implicated to function as a downstream target of NF-κB activation providing a link between these two signaling pathways.52 Our findings do not support the hypothesis that altered expression of Notch-1 or Jagged 1 may be involved in the photocarcinogenesis of SCCs or may be of importance for the growth characteristics of these tumors. Moreover, our findings do not support the hypothesis that pharmacologic modulation of Notch signaling, e.g., via vitamin D compounds, epigenetic drugs or other agents, could be a promising new target for the treatment of skin cancer. However, one has to keep in mind that our results are very limited and preliminary, and do not allow a definite conclusion on the role of Notch-1 or Jagged 1 for skin photocarcinogenesis. The importance of other factors that influence the role of Notch signaling for skin photocarcinogenesis, including the relevance of the microenvironment as an active contributor to tumor development by representing the primary source of proliferative signals to initiated cells, will have to be analyzed in future studies. These studies will help to clarify the role of Notch signaling for skin carcinogenesis.

Materials and Methods

Cell culture. Non-malignant (spontaneously immortalized HaCaT keratinocytes) and malignant (SCL-1 cells, derived from a cutaneous squamous carcinoma) human keratinocytes were maintained in RPMI 1640 medium (PAA Laboratories) supplemented with 10% fetal calf serum (Biochrom AG) and 1% L-glutamine, as reported previously.40

Cells were treated with UV B (UVB), 1,25(OH)2D3 (Sigma-Aldrich), trichostatin A (TSA, Sigma), and 5-azacytidine (5-Aza, Sigma) in various combinations, in various concentrations, and at different time points. 1,25(OH)2D3 and TSA were dissolved in ethanol as a stock solution (4 mM and 1 mg/ml, respectively) and stored in the dark in -20°C, 5-Aza was dissolved in PBS (10 mM). Media containing varying concentrations of 1,25(OH)2D3, TSA or 5-Aza and their combinations were added to cultured cells 24 h after seeding cells in tissue culture dishes (Greiner Bio-One GmbH), immediately after UV B-irradiation. In experiments involving 1,25(OH)2D3, media containing 1,25(OH)2D3 were added to cultured cells 24 h before or immediately after UV B-irradiation. Cells cultivated with medium alone or cells treated with vehicle (ethanol) alone served as controls.

UV-B irradiation. Immediately before irradiation with a UV-B-lamp (Waldmann), cells were washed with PBS. Cells were then irradiated (0 J, 100 J or 500 J) through a thin film of PBS. UV-B doses were determined using a dosimeter (Waldmann). Twenty-four hours before or immediately after irradiation, cells were provided with fresh medium containing various supplements as outlined above.

Proliferation analysis. The cells were plated into 24-well plates. Proliferation of untreated and treated cells was quantified by crystal violet (CV) dye staining, as reported previously.40 In short, the cells were washed once with PBS and fixed with ethanol (70%) for 30 min at room temperature. The cells were then incubated with a CV solution (1% in 20% ethanol) for 30 min at room temperature and rinsed with water thoroughly. After drying, the dye was extracted with 70% ethanol and its absorbance determined at 550 nm using a microplate reader (Titertek Multiskan Plus MK II, Labsystems).

Protein gel blotting. For protein isolation, cells were lysed in 50 µl of a lysis buffer heated to 100°C and containing 50 mM Tris–HCl (pH 6.8), 100 mM DTT, 2% SDS and 20% glycerol. Samples containing 5 µg of total cellular protein were subjected to 10% SDS–PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore). Signals were detected upon overnight incubation of the membranes with primary antibodies [directed against Notch 1 (EPITOMICS, Burlingame, #1935-1), jagged 1 (clone H-114, Santa Cruz Biotechnology, Inc., sc-8303) or GAPDH (clone FL-335, Santa Cruz Biotechnology, Inc., sc-25778)] followed by a final incubation with a peroxidase-conjugated secondary antibody and Renaissance Enhanced Luminol Reagents (NEN), performed as specified by the supplier.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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