Notch1 Can Contribute to Viral-Induced Transformation of Primary Human Keratinocytes

Stéphanie Lathion, Janina Schaper, Peter Beard, and Kenneth Raj

Swiss Institute for Experimental Cancer Research (ISREC) and National Center of Competence in Research (NCCR) Molecular Oncology, Epalinges, Switzerland

ABSTRACT

The human papillomavirus (HPV) is the most significant causative agent in the development of cervical cancer. Despite its presence in almost all cervical cancers, HPV by itself is unable to transform a normal cell to a cancerous one. Instead, additional cellular mutations are required to supplement the HPV oncoproteins E6 and E7. Activation of the Notch1 signaling pathway has been proposed as one of the cellular changes that cooperate with the E6 and E7 proteins to cause cervical cancers. This proposition is based on: (a) the detection of active Notch1 in high-grade cervical lesions and cancers; (b) the synergism between Notch1 and E6 and E7 to transform immortalized cells; and (c) the obliteration of neoplastic properties of a cervical cancer cell line when Notch1 expression was inhibited. However, this view was put in doubt by a recent report that showed Notch1 expression is markedly reduced in cervical cancer cells, and this was attributed to the ability of Notch1 to repress the expression of the HPV E6 and E7 proteins. Here we report that although exaggerated levels of Notch1 can, indeed, adversely affect HPV E6 and E7 expression, and cellular proliferation in general, moderate levels of Notch1, together with active phosphoinositide 3 kinase, can, instead, exhibit oncogenic properties that transform primary cells containing HPV16 E6 and E7 proteins. In addition, we show that activated Notch1 is readily detected in all cervical cancer cell lines tested. Together, these results show that not only do cervical cancer cells express Notch1, but also that Notch1 signaling, in synergy with other cellular changes, can participate in the transformation of primary cells expressing E6 and E7 proteins.

INTRODUCTION

An outstanding feature of cervical cancer is the presence of integrated copies of human papillomavirus (HPV) DNA in the genome of the cancerous cells (1). The correlation between the presence of HPV DNA and cervical cancers can be as high as 99% (2). Invariably, some copies of the integrated viral DNAs express two oncoproteins, the E6 and E7. Together, these proteins target two key control elements of cellular proliferation, the pRb protein and telomerase. The active pRb protein, in a complex with E2F transcription factor, inhibits expression of S-phase genes. Active pRb is also efficiently bound and inactivated by the HPV E7 protein. The result of this binding is the abrogation of transcriptional repression by pRb, and the subsequent expression of proteins required for progression in the cell cycle (3). The catalytic subunit of the telomerase complex, telomerase reverse transcriptase, is normally expressed in a tightly regulated fashion in the epithelium, it is higher in the membrane and cytoplasm of cells of precancerous cervical lesions (CIN II and CIN III). Importantly, in cervical cancers, the Notch1 protein was detected at high levels in the nucleus, indicating that the Notch1 was in the active, cleaved form. These observations led to the deduction that activation of Notch1 signaling may be an important step in the development of cervical cancers. This view was further strengthened by experiments showing that the continued presence of Notch1 is necessary for the maintenance of the neoplastic phenotype of a cervical cancer cell line, CaSki. These results, obtained by both gain and loss of function experiments, imply the active involvement of Notch1 signaling in cervical cancers. However, doubt was cast on this notion by Talora et al. (13), who reported that invasive and metastatic cervical cancers do not express Notch1 and that cell lines that were derived from HPV-induced cervical cancers were devoid of the Notch1 protein. This, they argued, is the consequence of the ability of Notch1 to repress the transcription of the E6 and E7 genes, and, hence, Notch1 expression would be selected against in the process of cervical tumorigenesis. According to this report, Notch1 would prevent, rather than aid, the process that leads to cervical cancer. Also, previous studies showed that Notch1 can function as a tumor suppressor in the skin of mice.

Overexpression of Notch has also been reported for endometrial and head and neck carcinomas (7, 8, 16, 17). In addition, Notch overexpression was initially detected in blood-related cancers such as
human T-cell acute lymphoblastic leukemia (18), Hodgkin and large cell anaplastic lymphomas (19). Hence, strong evidence exists to support both views: that Notch1 aids in the process of tumorigenesis and that Notch1 prevents tumorigenesis. With the situation being far from clear, we determined the status of Notch1 signaling in cervical cancer cells and tested whether Notch1, in human epithelial cells, inhibits or stimulates the process of transformation.

MATERIALS AND METHODS

Cells, Viruses, and Plasmids. All of the cells used in the experiments described, except for primary human foreskin keratinocytes (FSKs) and W12 and S12 cells, were cultured in 10% FCS in DMEM. W12 and S12 cells were cultured in medium that was made up of three parts Ham F-12 and one part DMEM, supplemented with 5% FCS, 8.4 ng/ml cholera toxin, 5 μg/ml insulin, 24.3 μg/ml adenosine, 0.5 μg/ml hydrocortisone, and 10 ng/ml epidermal growth factor. W12 cells were cultured in the presence of lethally irradiated NIH3T3 cells. Primary human FSKs were isolated from healthy donors by Dr. M. Huber, Dermatologic Service, University Hospital, Lausanne, Switzerland. Primary keratinocytes and immortalized derivatives were cultured in a medium that we designated as KGM. This medium is composed of three parts DMEM (Life Technologies, Inc.), one part Ham F-12 medium (Life Technologies, Inc.), 10% fetal bovine serum, supplemented with 10 ng/ml epidermal growth factor (Peprotec), 0.5 μg/ml hydrocortisone, 24 μg/ml adenine, 5 μg/ml insulin, and 8.3 ng/ml cholera toxin. The primary cells were cultured in the presence of irradiated NIH3T3 cells and grown on plates coated with a solution made up of 100 μg/ml BSA (Biofluid), 32 μg/ml bovine collagen type 1 (Vitrogen), and 10 μg/ml human fibronectin (Calbiochem). All of the cells were cultured at 37°C with 5% CO2.

Amphotropic recombinant retroviruses were produced using the phoenix A packaging cell line obtained from Dr. Gary Nolan (Stanford University, Stanford, CA). The genes of interest were cloned either into the pcDNA6 vector (Invitrogen) or into the pBabe series of retroviral vectors, pBabe Neo, pBabe Puro, and pBabe Hygro. The E6/E7-encoding DNA was obtained using PCR with forward primers that anneal to the first 20 bases of the E6 gene and reverse primers that anneal to the last 20 bases of the E7 gene. The p110/CACAX cDNA was obtained from Dr. Julian Downward (Cancer Research UK, London, UK). The Notch1 IC cDNA was obtained from Dr. M. J. Bevan (University of Washington, Seattle, WA). Dr. F. Radtke (Ludwig Institute for Cancer Research, Lausanne, Switzerland) provided the hairy enhancer of split (HES-1) luciferase reporter plasmid. Phoenix A cells were transfected with the appropriate vectors using the calcium phosphate method, and 24–48 h later, medium containing retroviruses was harvested and was either used immediately or frozen as 1-ml aliquots. Primary FSK cells prepared as described above were seeded onto plastic dishes to obtain ~60% confluence the next day. Viral supernatants were mixed with Polybrene to a concentration of 10 μg/ml. The mixture was layered onto cells and incubated at 37°C for 4 h before fresh medium was added. Twenty-four h later, cells were trypsinized and divided into three plates in the presence of appropriate antibiotics. Generally, antibiotics killed all uninfected control cells in 4 days. C4-1 cells were transfected with Transfast (Promega) reagent and HeLa cells with LipofectAMINE 2000 (Invitrogen) reagent according to the manufacturers’ instructions. C-33 cells were transfected using the calcium phosphate method.

Western Blot Analyses. Medium was aspirated from the plate, and the cell monolayer was washed with 10 ml of PBS. Cells were harvested by scraping and were collected in a 1.5-ml vial. After centrifugation at 6,000 rpm in an Eppendorf microcentrifuge, the supernatant was discarded and the cell pellet was resuspended in 100 μl of methanol and 100 μl of methanol buffer for at least 4 h. The membrane was blocked in 5% nonfat milk in PBS containing 2% Tween 20 for at least 1 h. Antibody to Notch1 (Santa Cruz Biotechnology; Catalog SC-6014) was added at a dilution of 1:2,000 in 5% nonfat milk in PBS/Tween-20, and incubated with the membrane for at least 1 h in a roller tube before the membrane was washed three times with PBS/Tween-20. Secondary antibody conjugated to horseradish peroxidase (The Jackson Laboratory) was added to the membrane at a dilution of 1:5,000 in 5% nonfat milk in PBS/Tween-20 and was incubated at room temperature with gentle agitation. One h per wash, the membrane was washed with three changes of PBS/Tween-20, at 10 min per wash. Signals on the membrane were revealed with the enhanced chemiluminescence mixture purchased from Amersham. Light signals were captured on Kodak BioMax films. Typically, signals for Notch1 IC were detectable after 5 min of exposure of the membrane to the film.

Thymidine Incorporation Assay. Cells were first labeled with 15 nCi [3H]thymidine per plate during 24 h, after which the medium was removed and the cell monolayer washed twice with DMEM. The cells were then cultured in DMEM supplemented with 10% FCS for another day. After that 5 μCi of [3H]thymidine was added to the cells. After 30 min, the medium of the cells was removed, and the cell monolayer was washed twice with PBS and was trypsinized. The cells were centrifuged at 2000 rpm in a tabletop centrifuge, and the supernatant was discarded. The cell pellet was washed once with 10 ml of PBS and was centrifuged as described above. The cell pellet was then fixed with cold methanol for at least 2 h, the cells were centrifuged, the methanol was discarded, and the pellet was resuspended in methanol and centrifuged. The cell pellet was resuspended in 200 μl of methanol and spotted onto GFC filters (Whatman). After air-drying, the filters were immersed in a scintillation cocktail (Beckman), and the radioactivity was measured in a Beckman counter. The 14C and 3H counts were corrected for cross-channel overflow. The 14C count was used to standardize the amount of input DNA (hence, the amount of cells), and the 3H counts revealed the rate of DNA synthesis.

Luciferase Reporter Assay. Cells were transfected with a mixture of plasmids expressing the Notch1 IC protein (either pcDNA6 Notch1 IC or pBabeHygro Notch1 IC), plasmids expressing renilla luciferase, and plasmids with wild-type or mutant enhancer or with promoter of HPV18 (from Drs. F. Hoppe-Seyler and T. Banknecht, German Cancer Center, Heidelberg, Germany) cloned upstream of a firefly luciferase gene. Forty-eight h later, cells were harvested by scraping, and the cell pellet was resuspended in 100 μl of Reporter Lysis Buffer (Promega). After 30-min incubation, the supernatant was collected, the protein concentration was assayed as described above, and the extract was assayed for the activity of firefly and renilla luciferase using a kit obtained from Promega. The renilla luciferase activity serves as an indicator of transfection efficiency, and the firefly luciferase activity reveals the extent by which the HPV enhancer and promoter is affected by the Notch1 IC protein.

Soft Agar Assay. For soft agar assays, bottom layers of 0.5% Bacto-agar (Difco) in KGM were prepared in 6-cm plates. Cells were harvested by trypsinization and were counted and seeded in triplicate with 200, 2,000, or 20,000 cells per 6-cm dish in a 0.3% Bacto-agar top layer. The cells were fed with KGM every 2 days. HeLa and NIH3T3 cell lines were used as positive and negative controls, respectively, under similar culture conditions, with the exception that the medium used was DMEM (Life Technologies, Inc.). The dishes were incubated at 37°C for ~2 weeks.

Tumor Formation in Nude Mice. Cells were trypsinized, counted, and adjusted to a concentration of one million cells in 50 μl of DMEM. Cells (50 μl) were injected under the skin of nude mice. Mice were checked every 3 days to monitor tumor formation. Tumor diameters were measured, and the volumes were calculated with the formula 4/3πr3.

RESULTS

Expression of Notch1 in Cervical Cancer Cell Lines. Cell lines derived from cervical lesions were tested for the expression of the Notch1 protein. These cells contained either HPV16 (SiHa, W12, and S12) or HPV18 (C4-1 and HeLa) DNA. Apart from W12 cells, all other cell lines were derived from cervical cancers and contain intact copies of the HPV genome. W12 cells were derived from a low-grade cervical lesion and contain episomal copies of HPV16 DNA (20). HaCaT and C-33 cells are HPV-negative keratinocytes. The results in Fig. 1 show that all of the cervical cancer-derived
HPV-containing cell lines that were tested possessed readily detectable levels of the Notch1 protein. In addition, the size of the Notch1 proteins detected (M_r ~110,000) corresponds to that of the processed form, the Notch1 IC. There appears to be no correlation between HPV presence or absence, and Notch1 expression. Interestingly, S12 cells (21), which are derived from the W12 cells, expressed high levels of Notch1 protein, whereas W12 cells expressed very low or undetectable levels of the protein. The S12 cells, like the cell lines derived from cervical cancers, contain integrated HPV16 DNA and can grow rapidly and autonomously, whereas the W12 cells, like cells of low-grade cervical lesions, possess episomal (un-integrated) HPV DNA, require feeder cell support for growth and proliferate slowly. Hence in this isogenic pair of cell lines, endogenous protein levels of Notch1 IC are low in the slow-growing W12 cells but high in the fast-growing S12 cells.

The Effect of Notch1 on Cellular Proliferation. To test the effect of exogenously expressed Notch1 on cellular proliferation, two HPV18-containing cell lines, HeLa and C4-I, and one HPV-negative cell line, C-33, were transfected with a vector that expresses activated Notch1 (Notch1 IC) under the control of a cytomegalovirus (CMV) early promoter. The Notch1 IC protein was fused to a c-myc tag at the COOH-terminus. Fig. 2A shows an immunoblot of protein extracts from cells that were transfected with this vector, probed with antibodies against the c-myc tag. It is clear that this vector expresses exogenous Notch1 IC to high levels. Transfected cells were selected with the appropriate antibiotics for 2 days and were subjected to thymidine uptake experiments. The results in Fig. 2B show that exogenously expressed Notch1 IC from a CMV promoter severely reduced incorporation of thymidine by HPV-containing cells, indicating either that DNA replication was retarded or that fewer cells were undergoing DNA synthesis compared with control cells. In either case, cellular proliferation is reduced. Importantly, a reduction was also observed in the HPV-negative C-33 cells, albeit to a lesser extent. Hence, it can be concluded that, although Notch1 IC inhibits proliferation of HPV-containing cells, it is doing this via a route that can also affect the cell independently of HPV.

The Effect of Notch1 on the Early Promoter of HPV. To ascertain whether the suppression of proliferation of HPV-containing cells by Notch1 IC is mediated through reduction of E6 and E7 expression, cells were transfected with the CMV-driven Notch1 IC-expression vector and selected with appropriate antibiotics, and the relative levels of E6 and E7 transcripts were compared. The exogenously expressed Notch1 IC exerted a slight but consistent reduction in the levels of the E6 and E7 RNAs (Fig. 3), suggesting that Notch1 might repress the activity of the HPV early promoter, which controls expression of E6 and E7. This was further tested by quantitative measurements of luciferase activity in transient transfection assays. Plasmids bearing the HPV18 promoter and enhancer region [the upstream regulatory region (URR)] were coupled to the luciferase gene. Plasmids with the wild-type HPV URR sequence, or plasmids bearing mutations at various regions of the enhancer or promoter (22, 23), were used in the experiments. These mutations were at sites that are recognized by major transcription factors, namely keratinocyte regulatory factors, glucocorticoid responsive elements, activator protein 1 (AP1), and Yin Yang 1 (YY1). From Fig. 4, it is clear that mutations in all of these sites on the URR resulted in decreased basal activity of the HPV early promoter. Interestingly, when basal activities of all of the URRs were normalized to 100%, it becomes apparent that Notch1 IC repressed the activity of all of the URRs (wild type and mutants) to a similar extent. The results show that repression of the HPV promoter by Notch1 IC is not mediated by keratinocyte regulatory factor.
glucocorticoids, or YY-1. Importantly, two constructs with AP1 recognition sites mutated in either the promoter (AP1-p) or the enhancer (AP1-e) region were so severely affected that the basal activity of these URRs were reduced by more than 90%. Luciferase activity from the URR with a mutation at the AP1-e was reduced to that of background, and, hence, the effect of Notch1 on this URR was not verifiable. Clearly, the AP1 sites, especially AP1-e, are of fundamental importance for the activity of the HPV promoter. Because all of the HPV promoter constructs that were affected by Notch1 IC contained an AP1 site, our results are consistent with Notch1 signaling repressing the HPV promoter activity via AP1, although we have no direct evidence for it. This notion is supported by the results of Talora et al. (13), who showed that Notch1 signaling reduces the levels of AP1 in the cell.

The Western blot of Fig. 1 shows endogenous Notch1 in the active form, the Notch1 IC. Clearly at this level, Notch1 neither suppresses the proliferation of cells nor inhibits E6 and E7 expression. Hence, the question raised by these results is whether the effects of Notch1 IC on cellular proliferation and the HPV promoter are due to the exaggerated levels of exogenous Notch1 IC expressed in the cells. To address this question, cells were transfected with another construct bearing the same Notch1 IC DNA sequence under the control of a weaker promoter, the Moloney murine leukemia virus long terminal repeat (LTR).

Fig. 3. Northern blot analysis of transcripts from HeLa cells or HeLa cells transfected with pcDNA6 Notch1 IC. The blot was probed with 32P-probes against HPV18 E6 and E7 or β-actin.

Fig. 4. Measurement of HPV18 promoter activity by luciferase reporter assay. HeLa cells were transfected with luciferase constructs bearing either wild-type (WT) or mutant (mut) HPV18 upstream regulatory region, together with pcDNA6 Notch1 IC. Top graph, absolute luciferase values; bottom graph, percentage change of luciferase values. AP1-p, activator protein 1 promoter; AP1-e, activator protein 1 enhancer; KRF, keratinocyte regulatory factor; GRE, glucocorticoid responsive element; YY1, Yin Yang 1.

Fig. 5. A, thymidine incorporation assay of HeLa cells expressing intracellular Notch (Notch1 IC) from a retroviral long terminal repeat (LTR) promoter. B, measurement of HPV16 or HPV18 promoter activity by luciferase reporter assay. HaCaT cells were transfected with luciferase constructs bearing the human papillomavirus (HPV) promoter, together with a vector expressing Notch1 IC from the LTR promoter. Top, percentage change in luciferase values caused by Notch1 IC; bottom, comparison of percentage changes in luciferase values induced by Notch1 IC expressed either from the retroviral LTR or from the cytomegalovirus (CMV) immediate early promoter. URR, upstream regulatory region. C, measurement of the effect of Notch1 IC expressed at low (from the LTR promoter) or high (from the CMV promoter) levels on the Notch1 IC target, the HES-1 promoter in HaCaT cells. Exp., experiment; HES-1, hairy enhancer of split.
promoter. Proliferation of transfected cells was analyzed and found to be only marginally affected (Fig. 5A). Likewise activities of the HPV16 and HPV18 promoters were only very slightly altered (Fig. 5B), and transcription of E6 and E7 was not noticeably changed (data not shown). Importantly, Notch1 IC was indeed expressed from the LTR promoter in cells, because it was able to activate the HES promoter, a known downstream target of Notch1, as efficiently as could Notch1 IC that was expressed from the CMV promoter (Fig. 5C).

**Notch1 IC Contributes to the Process of Tumorigenesis of Primary Keratinocytes.** Having established that Notch1 signaling, at levels that are not exaggeratedly high, does not inhibit cellular proliferation, we asked whether Notch1 IC at moderate levels could instead contribute to tumorigenesis. In this study, the potential contribution of the constitutively active p110α subunit of the phosphoinositide 3 kinase (PI3K) complex, the p110αCAAX (24), was also tested in parallel. PI3K activity was reported to be consistently higher in cervical cancer cells than in normal cells (25). This is consistent with the finding that the DNA locus that harbors the p110α gene is amplified in up to 90% of cervical cancers tested (26, 27). Primary human FSKs were infected with retroviruses bearing the HPV16 E6 and E7 genes. After selection with the appropriate antibiotics, cells were infected with either Notch1 IC-bearing retroviruses or p110αCAAX-bearing retroviruses, or both, followed again by selection with appropriate antibiotics. All of the various cell populations generated (FSKE6/7, FSKE6/7 + Notch1 IC, FSKE6/7 + p110αCAAX, and FSKE6/7 + Notch1 IC + p110αCAAX) were harvested and seeded in soft agar. After 2 weeks of incubation, pictures of the colonies were taken (Fig. 6A), and colonies were scored (data not shown). Whereas FSKE6/7 cells failed to grow efficiently in soft agar, with the exception of a few sporadic colonies, FSKE6/7 + Notch1 IC cells formed small colonies in the agar, FSKE6/7 + p110αCAAX cells, on the other hand, did not form very many more colonies than FSKE6/7 cells, but the colonies were clearly larger. Interestingly, FSKE6/7 + Notch1 IC + p110αCAAX cells formed more and larger colonies in the agar. These colonies were comparable with those formed by HeLa cells. However, on closer examination (Fig. 6B) it is clear that, whereas the colonies of FSKE6/7 + Notch1 IC + p110αCAAX cells appeared compact, those of HeLa cells appeared rather loose. It is not known what is responsible for this difference and whether it is related to differences observed in tumor induction. Whereas HeLa cells injected under the skin of nude mice formed large viable tumors, FSKE6/7 + Notch1 IC + p110αCAAX cells formed small tumors with a maximum diameter of 6 mm, which regressed after 10 weeks (data not shown). It is evident that cellular changes in addition to those induced above are required to further transform the cells to a state in which they can form continuously growing tumors under the skin of nude mice.

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**Fig. 6. A**, assay for the ability of cells to grow independently of physical attachment to a substrate. Cells were seeded in soft agar and were cultured for ~2 weeks. *Pictures of cells on the bottom row are at a 4-fold higher magnification than those in the top row. B*, comparison of the morphology of a colony derived from foreskin cells expressing HPV16 E6/7, p110αCAAX, and intracellular Notch (Notch1 IC) with one derived from HeLa cells. FSK, human foreskin keratinocyte.
Nevertheless, the results are clear that Notch1 IC can, together with E6 and E7 proteins and cellular changes such as PI3K activation, exhibit oncogenic properties.

DISCUSSION

The possible role of Notch1 in cervical cancers was first proposed by Zagouras et al. (8) based on their observation that Notch1 is highly expressed in cervical cancers. Daniel et al. (7) noted that Notch1 expression increases with the severity of HPV-containing cervical epithelial lesions. In the most severe stage, Notch1 staining was nuclear, indicating that not only were the levels of Notch1 protein high but that they were active as well. Rangarajan et al. (10) later supported this work by showing that expression of Notch1 IC together with HPV oncogenes transformed HaCaT cells, a spontaneously immortalized epithelial cell line. Importantly, Weijzen et al. (11, 12), showed by using inducible antisense RNA, that Notch1 expression is required for the maintenance of the neoplastic phenotype of an HPV16 DNA-containing cervical cancer cell line, CaSki. As persuasive as these observations are, a clear lack in the evidence is the demonstration that the induction of Notch1 signaling, either alone or in cooperation with other cellular changes, contributes to the transformation of primary cells. Here we report such an observation, and we address the apparently opposing properties of Notch1 signaling.

That Notch1 signaling might be important in cervical tumorigenesis was first tested by determining whether Notch1 was evident in cells derived from cervical cancers. Notch1 protein was indeed found in these cells, and importantly, the active form of Notch1, the Notch1 IC, was easily detectable. This observation does not prove the importance of Notch1 signaling, but it questions the conclusion that Notch1 protein expression is reduced or absent in cervical cancers (13). Intriguingly, some of the same cell lines were used in both studies. Although we cannot account for the discrepancy, we are aware that other groups, Weijzen et al. (11, 12) and Krishna et al., have also detected Notch1 IC protein in HPV-containing cervical cancer cell lines. The only cell in which Notch1 IC was not detected was the W12 line, which was derived, not from a cancer of the cervix, but from a low-grade cervical lesion (20). It is also the only cell line among the panel of cells tested that harbors the HPV DNA in the episomal (nonintegrated) state. Interestingly, a cell line derived from W12, called S12 (21), expressed high levels of Notch1 IC. The S12 cells used in these experiments no longer harbor episomal HPV16 DNA. Instead, the viral DNA is integrated into the host genome, as in cervical cancer-derived cell lines. Whereas the growth of W12 cells requires fibroblast feeder support, S12 cells do not. In addition, S12 cells proliferate significantly faster than do W12 cells. The cause of elevated Notch1 expression in S12 cells is yet to be addressed, but it might be due to increased E6 and E7 expression in S12 cells. Weijzen et al. (12) reported that the E6 and E7 proteins, by acting at various levels of gene expression, could increase Notch1 IC protein levels in the cells. E6 and E7 expression is higher in S12 cells than in W12 cells, as we have confirmed (data not shown) and as was reported for similar isogenic lines by Jeon et al. (28). This question, although interesting, is not within the scope of this report. Results from the pair of isogenic cell lines, W12 and S12, nevertheless, show that activation of the Notch1 signaling pathway is not correlated with reduced cellular proliferation. Instead, the activation appears to correlate with increased cellular proliferation, a feature that seems to be at odds with the notion of Notch1 signaling being antiproliferative.

When the effect of exogenously expressed Notch1 IC on cells was tested, we observed that cellular proliferation was severely inhibited. Such inhibition was clear in E6- and E7-containing cells and is still evident in cells that lack HPV. This implies that exogenous Notch1 IC can impede cellular proliferation via a route that is general to cells but that is particularly crucial to E6- and E7-expressing cells. This is also supported by the results of Talora et al. (13), who showed that recombinant adenovirus expressing Notch1 IC represses the HPV promoter that controls HPV E6 and E7 transcription.

Whereas our studies using wild-type and mutant HPV18 URR-luciferase constructs do not conclusively reveal how Notch1 represses the HPV early promoter, they showed that the keratinocyte regulatory factor, glucocorticoid, and YY1 transcription factors were not important in this respect. The relevance of AP1 sites is of particular interest. The AP1 sites are present in all of the other mutant HPV18 URRs tested, and Notch1 IC affected the activity of all these mutants to a similar extent. Although this suggests, not proves, the involvement of AP1, it does fit with the suggestion of Talora et al. (13), who showed that the repression of the HPV promoter by Notch1 can be relieved by the forced expression of the c-Fos subunit of the AP1 (Fos-Jun) heterodimer, providing a strong reason to propose that exogenously expressed Notch1 IC reduces AP1 levels in the cells. Such an effect would be expected to reduce E6 and E7 levels, which have been previously shown to be largely dependent on AP1 (29, 30). Also implicit in this proposal is that, because of the importance of AP1 in the transcription of many cellular genes, Notch1 would also affect cells lacking HPV, albeit to a lesser extent. This expectation was met in the thymidine uptake experiment described above. Although Talora et al. (13), conclude that Notch1 IC expression repressed DNA synthesis only in cells that contain HPV, this seems inconsistent with the observations of Rangarajan et al. (14), who showed, using the same reagents, that DNA synthesis in primary mouse keratinocytes was suppressed by high levels of Notch1 IC.

It appears that whereas endogenous Notch1 expression is not detrimental to cellular proliferation, exogenously expressed Notch1 IC is. The possibility that the exogenously expressed Notch1 IC was mutant was excluded by verification of the DNA sequence (data not shown). Because the level of exogenously expressed Notch1 IC from a CMV promoter is high, and the endogenous Notch1 IC level is already rather elevated in these cells (as shown in Fig. 1), we asked whether the effect of exogenous Notch1 IC on cells is due to exaggerated levels of the protein. The answer is clearly affirmative. When Notch1 IC was expressed from the retroviral LTR (a promoter weaker than CMV), there was hardly any effect on cellular proliferation or E6 and E7 expression, although the retroviral LTR expressed sufficient Notch1 IC protein to stimulate the HES promoter, a known Notch1 target. These results imply that the activation of Notch 1 signaling in cells per se does not inevitably reduce the proliferation of HPV-containing cells. Instead, it is the substantial increase of the Notch1 IC level over that required for activation of Notch-responsive promoters that elicits repression of the HPV promoter and inhibition of cellular proliferation. Whether cells in vivo ever experience such high levels of Notch1 IC is a crucial question in clarifying the role of Notch1 in cervical tumorigenesis. The difference between normal cells and cancer cells may not be characterized by big changes in expression of oncogenes or tumor suppressor genes but, rather, in subtle changes in numerous genes, at least in the case of colon cancer (31, 32).

When Notch1 IC was expressed at moderate levels in cells, it could, together with E6, E7, and active PI3K, induce the efficient growth of cells in soft agar, a test of cellular transformation, and of small tumors (that regressed) under the skin of athymic mice. We further observed that FSKE6/7 cells, transfected with CMV-driven Notch1 IC, expressed high amounts of the protein the first 3 days, but when the transfectants were analyzed after a week of growth, the amounts of

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1 S. Krishna, personal communication.
Notch1 IC were diminished sharply, presumably because of the negative selection of cells that express high amounts of Notch1 IC and/or because of the silencing of the promoter. In any case, these cells formed colonies in soft agar and tumors (again, that eventually regressed) in nude mice, just like FSK6/7 cells that contained the LTR-driven Notch1 IC (data not shown). These results show that expression of low levels of Notch1 IC in FSK6/7 cells, regardless of the type of promoter used, can exhibit tumor-promoting characteristics.

Two cellular changes that are frequently observed in cervical cancers, the activation of Notch1 and of PI3K, act in synergy to induce anchorage-independent growth of E6 and E7-expressing primary cells. The number of colonies formed was only slightly lower than that formed by HeLa cells. Interestingly, whereas colonies formed by cells expressing Notch1 IC, PI3K, E6, and E7 (FSK6/7 + Notch1 IC + PI3K) appeared compact, colonies formed by HeLa cells were loose, and individual cells could be visualized. These individual cells migrated away from the colonies and established new colonies, accounting for a higher number of HeLa colonies in the agar than the number of HeLa cells seeded. The FSK6/7 + Notch1 IC + PI3K cells did not make this excess of colonies, but 80% of the seeded cells formed colonies, a result that attests to the efficiency of Notch1 IC and PI3K in conferring this property on FSK6/7 cells. However, full transformation of these cells was not attained because they failed to persist as tumors when injected under the skin of nude mice. The inability of FSK6/7 + Notch1 IC + PI3K tumors to persist is most likely due to a need for additional cellular changes. This point is being addressed, but it does not alter the fact that Notch1 IC, at moderate levels, can contribute to the process of cellular transformation.

This raises the conundrum of the opposing properties that the Notch1 can exhibit. The explanation made thus far is based solely on the levels of Notch1 IC. However, Nicolas et al. (15) showed that Notch −/− mice develop spontaneous tumors in the skin. Such observations argue that Notch1 can behave like a tumor suppressor. Yet, in our experiments, Notch1 functions more like an oncoprotein. Although such results apparently seem conflicting, proteins with dual properties that are opposing in nature are, nevertheless, not new. In this respect, Notch1 is preceded by c-myc and ras, two oncoproteins whose cell-type-specific activity of the human papillomavirus type 18 upstream regulatory promoters and PKB/Akt-mediated survival in Hodgkin lymphoma revealed by the use of laser capture microdissection and cDNA arrays. Onco- genesis, 19: 3220–3224, 2000.


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Stéphanie Lathion, Janina Schaper, Peter Beard, et al.


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