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Alterations in Notch signaling in neoplastic lesions of the human cervix

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ABSTRACT **The development of cancer is a cellular process that reflects and is partly driven by alterations in cell determination. Mutations in various molecules responsible for cell determination have been identified as being oncogenic, but little is known about the involvement of normal cell fate-determining mechanisms in the oncogenic process. The Notch pathway defines an evolutionarily conserved, general cell interaction mechanism that controls fundamental aspects of cell determination during vertebrate and invertebrate development. We have explored the involvement of the human Notch pathway in human cervical tissues, which define a cellular environment where cell fate changes take place and where neoplastic conditions have been well characterized. Our evidence suggests that Notch expression is associated with cell populations that are undergoing cell fate changes and that Notch activity can be used to monitor cell fate abnormalities in cervical as well as other epithelial neoplasias.**

Human adult epithelia are renewed from a reserve of immature stem cells that, in response to developmental signals, take on specific fates in replenishing mature tissues (1). The unfolding of the developmental program depends on the cells' ability to receive and interpret these signals. The accumulation of somatic mutations in stem cells can result in various pathological conditions, including neoplasias. Indeed, from a developmental point of view, neoplastic cells are cells that proliferate and differentiate out of the normal context. While much effort has been devoted to defining the genetic changes that can lead to a cancerous state, little is known about the role that cell determination molecules play in cancer. Genetic analysis of *Drosophila* development, however, has identified several cell interaction pathways that control fundamental aspects of cell differentiation. Comparative analyses have demonstrated a remarkable degree of functional conservation between many developmentally important *Drosophila* genes and their vertebrate counterparts. An evolutionarily conserved cell interaction mechanism that plays a fundamental role in controlling the progression of immature cells to a more differentiated state involves the Notch locus. The activity of the Notch protein, a transmembrane receptor, controls most regulative decisions in *Drosophila.* We therefore decided to investigate the involvement of this cell interaction mechanism in the differentiation of human epithelia under normal and neoplastic conditions.

The Notch locus encodes a large transmembrane protein with an ≈1700 amino acid extracellular domain containing 36 epidermal growth factor-like repeats and an intracellular region of about 1000 amino acids containing 6 ankyrin repeats (2, 3). Notch acts as the receptor in a signaling pathway that includes two membrane-bound Notch ligands as well as cytoplasmic and nuclear elements identified through genetic and molecular analyses (reviewed in ref. 4). Activation of the Notch receptor may block or delay the progression of immature cells toward a more committed state (5–11). Furthermore, inactivating Notch in a cell that normally expresses it also leads to the acquisition of an incorrect fate (12–14). Thus, modulation of the Notch pathway may provide a general way to influence the fate of developmentally immature cells.

In *Drosophila*, there is a good correlation between Notch expression and cells that are not terminally differentiated. Cell populations that may be determined but have not yet acquired their final differentiation state, such as the proliferative epithelia of imaginal discs, express high levels of Notch (15). In contrast, almost all adult tissues are devoid of Notch expression, with the exception of ovaries and testes (16), which are the only adult tissues containing immature cells. *In situ* hybridization analyses involving Notch homologues in vertebrates also have established a correlation between Notch activity and undifferentiated, proliferative cell populations (17). Given the general involvement of Notch in the differentiation of immature cells in invertebrates and vertebrates, we were interested in examining Notch expression in adult human epithelia, which, unlike *Drosophila* epithelial tissues, include proliferative, immature cell populations. Cervical tissues appeared to be well suited for this purpose since they contain squamous and columnar epithelia that are constantly renewed from precursor cells, and frequently the columnar epithelium converts into squamous epithelium (squamous metaplasia). In addition, neoplasias of the cervical epithelia are common and are morphologically well characterized. Thus, the cervix provides a good system to examine Notch activity in a tissue that displays a diverse array of cell fate changes.

MATERIALS AND METHODS

Cloning and Antibody Production. Notch 1 and Notch 2 were cloned from a human fetal brain library (18). Notch 1 was found to be almost identical to the published sequence of TAN-1 (19). The complete sequence of the genes will be published elsewhere. The least conserved regions of the Notch 1 and Notch 2 intracellular domains, encoded by nucleotides 6658–7131 and 6508–6906, respectively (18), were PCR amplified. The PCR products were concatamerized (three copies) and cloned into the *Bam*HI site of the pGEX-2X vector (Pharmacia). The fusion proteins were produced in bacteria (20) and used to immunize rats. The immunized rat spleen cells were then fused to mouse myeloma cells for the production of hybridomas according to standard procedures. The hybridoma supernatants were tested by immunofluorescence and Western blot on cells transfected with plasmids encoding the cytoplasmic domains of Notch 1 or Notch 2. The polyclonal antiserum PGHN was raised against a different nonconserved region of Notch 2. A PCR-amplified fragment corresponding to nucleotides 6751–6906 was cloned into the pGEX-2X vector as a

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Abbreviation: BHK, baby hamster kidney.
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concatamer (nine copies). The fusion protein was injected into rabbits for the production of the polyclonal antiserum. The serum was tested by immunoprecipitation and Western blots on lysates of cells transfected with plasmids encoding the intracellular domain of Notch 2.

Expression, Immunofluorescence, Immunoblotting, and Immunoprecipitation. The full-length Notch 1 and Notch 2 cDNAs were cloned into the expression vector pcDNAI/Amp (Invitrogen). The specificity of the antibodies was tested by staining baby hamster kidney (BHK) cells transfected with the plasmids as described (21) using the vaccinia T7 system (22). The cells were fixed with 3% paraformaldehyde, washed, and incubated overnight with a 1:10 dilution of a hybridoma supernatant. After washing with phosphate-buffered saline (PBS), the cells were incubated for 1 hr with a 1:1000 dilution of Cy3-conjugated goat anti-rat antibody (Jackson ImmunoResearch) in PBS.

For immunoblotting, the transfected cells were lysed in Laemmli buffer containing 50 mM dithiothreitol and subjected to SDS/PAGE electrophoresis on 3–15% gradient gels. After transfer to nitrocellulose, the blots were incubated with hybridoma supernatants at a 1:10 dilution, followed by incubation with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rat secondary antibody (The Jackson Laboratory). For immunoprecipitation, cells were labeled with 50 μCi of [^{35}S]methionine per ml (1 Ci = 37 GBq) for 15 min and then incubated in medium containing excess unlabeled methionine for the indicated times. Cells were lysed in a detergent solution (300 mM NaCl/50 mM Tris, pH 8.0/0.5% Nonidet P-40/0.5% sodium deoxycholate/1 mM $CaCl_2$/1 mM $MgCl_2$) containing proteinase inhibitors and the lysates were precleared with normal rabbit serum. Then SDS was added to the supernatants at a concentration of 0.1% followed by overnight immunoprecipitation at 4°C with the polyclonal antiserum PGHN. The immunoprecipitates were washed in RIPA buffer and analyzed by SDS/PAGE on 3–18% gels followed by fluorography.

Immunohistochemical Staining. Surgical samples were fixed in formalin and paraformaldehyde and embedded in paraffin. Nonheated 5-μm sections were deparaffinized with xylene and endogenous peroxidase was quenched with H_2O_2. After blocking with a solution consisting of PBS, 0.1% Triton X-100, 5% normal rabbit serum, and 0.5% bovine serum albumin (BSA), the sections were incubated for 2–16 hr with primary antibody at 4°C in the same solution without BSA. Bound antibodies were detected using the Vectastain ABC kit (Vector Laboratories) using diaminobenzidine as peroxidase substrate (brown color in the photomicrographs). The sections were counterstained with Meyer's hematoxylin (blue color in the photomicrographs).

RESULTS AND DISCUSSION

We and others have reported isolation of human Notch homologues, TAN-1 (Notch 1) and hN (Notch 2) (18, 19), as well as four homologues of groucho (the TLE genes), a nuclear element of the Notch pathway that is encoded by the Enhancer of split complex (18). Homologues of both Notch genes have also been isolated from other vertebrate species (23–25). Antibodies recognizing the TLE proteins, and therefore permitting an expression analysis, have been described (18). To examine the expression of the human Notch proteins, antibodies were generated against the nonconserved areas of the cytoplasmic domains of the two Notch molecules. The specificity of each antibody was examined by indirect immunofluorescence and Western blot analysis of BHK cells transfected with expression constructs harboring either the Notch 1 or the Notch 2 gene. For the current study we selected antibody 15A, which recognizes Notch 1, and antibody 6D, which recognizes Notch 2. BHK cells were transiently transfected with a Notch 1 cDNA expression construct and stained with either antibody 15A or 6D. Cells expressing Notch 1 were detected only with antibody 15A (Fig. 1*A*) but not with antibody 6D (data not shown). Similarly, antibody 6D stained only cells expressing Notch 2 (Fig. 1*B*). In both cases the transfection was transient and therefore only a fraction (typically 30%) of the cells expressed a given construct. The remaining nontransfected cells served as an internal negative control. The transfected cell populations were also examined by Western blot analysis. Antibody 15A did not recognize any immunoreactive material in cells expressing Notch 2 construct or in mock-transfected cells (Fig. 1*C*, lanes 2 and 3). In contrast, 15A recognized

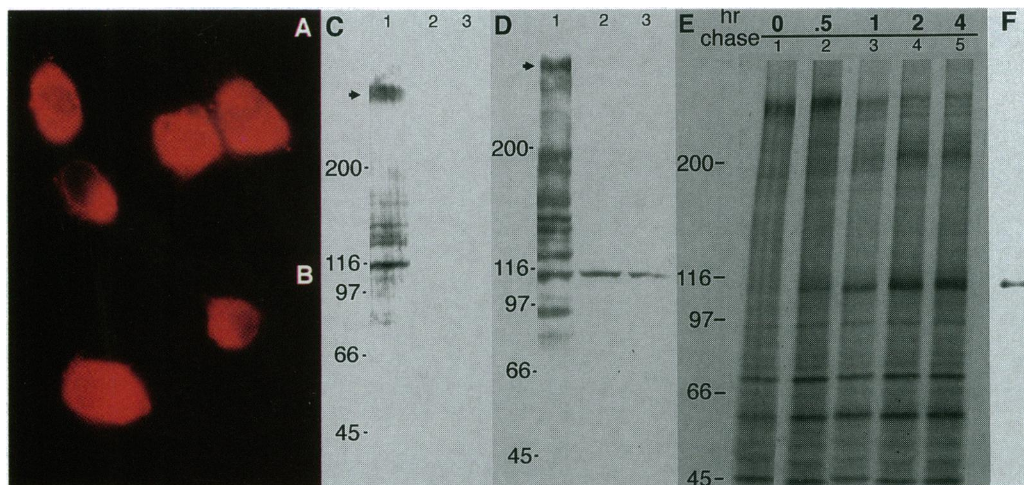

FIG. 1. Specificity of anti-Notch antibodies. (*A* and *B*) Immunofluorescent staining of Notch 1 and Notch 2 expressed in BHK cells. Cells were transfected with cDNAs corresponding to the full-length Notch 1 (*A*) and Notch 2 (*B*) genes. Protein expression was observed on the cell surface and intracellular membranes. Untransfected cells showed no staining. (*C* and *D*) Western blots of lysates from BHK cells expressing Notch proteins. Cells were transfected with cDNAs encoding Notch 1 (lane 1) or Notch 2 (lane 2) or were mock transfected (lane 3). After lysis, SDS/PAGE, and transfer to nitrocellulose, the blots were hybridized with antibody 15A against Notch 1 (*C*) or antibody 6D against Notch 2 (*D*). In addition to the full-length Notch proteins (arrowhead) that run as a 300-kDa species, bands of higher mobility were detected, corresponding to degradation products (lane 1). (*E*) Pulse–chase of NB5 neuroblastoma cells. Cells were labeled with [^{35}S]methionine for 15 min and then incubated in medium containing excess unlabeled methionine for the indicated times. The immunoprecipitates were analyzed by SDS/PAGE followed by fluorography. (*F*) Immunoprecipitation of Notch 2 protein from unlabeled NB5 cells. Cells were lysed and immunoprecipitated as in *E*. After SDS/PAGE and transfer to nitrocellulose, the blot was hybridized with the Notch 2-specific antibody 6D.

antigens when the cells expressed Notch 1 (Fig. 1*C*, lane 1). Similarly, antibody 6D could only recognize immunoreactive material in cells expressing the Notch 2 construct (Fig. 1*D*, lane 1). We note however, that 6D also recognizes a peptide in mock transfected cells as well as in cells expressing Notch 1 (Fig. 1*D*, lanes 2 and 3). We presume, but have not shown, that this ≈100-kDa band may reflect the expression of endogenous BHK Notch proteins that crossreact with our antibody. Taken together these results indicate that antibody 15A recognizes specifically human Notch 1 while antibody 6D is specific for human Notch 2. We have also generated a polyclonal rabbit antiserum, PGHN (see *Materials and Methods*), which in immunoprecipitations specifically recognizes Notch 2.

The Western analysis depicted in Fig. 1 shows that in addition to a polypeptide band corresponding to the full-length Notch product, several lower mass polypeptides are recognized by the antibody, presumably reflecting breakdown or processed Notch polypeptides. The most prominent species detected have a molecular mass of about 100 kDa. Such major Notch products are consistently seen in all human cell extracts we have examined so far and have also been detected in analogous studies in *Drosophila* (26).

We examined the relationship between the 100-kDa band and the full-length Notch by pulse–chase analysis of human neuroblastoma NB5 cells (27), which express endogenously Notch 1 and Notch 2. NB5 cells were labeled with [^{35}S]methionine and then incubated in medium containing an excess of unlabeled methionine for the indicated times. Cell lysates were immunoprecipitated with the polyclonal antiserum PGHN. The results are summarized in Fig. 1*E* and show that Notch is synthesized as a 300-kDa precursor (Fig. 1*E*, lane 1), which is rapidly processed to give a major product of 100 kDa (Fig. 1*E*, lanes 2–5). When unlabeled lysates of NB5 cells were immunoprecipitated with PGHN and then blotted with 6D, the only detectable band was in fact the 100-kDa processed form of Notch 2 (Fig. 1*F*), consistent with our earlier observations that at steady state the 100-kDa band represents the major Notch polypeptide species. These studies demonstrate a precursor/product relationship between full-length Notch and the 100-kDa Notch fragment.

The availability of specific Notch antibodies permitted examination of Notch expression in the dynamic cellular environment of the cervix under normal and pathogenic conditions. The vaginal part of the cervix is covered by a stratified squamous epithelium that is replaced every few days by a process of surface desquamation and regrowth from below. This epithelium is continuous with the simple columnar epithelium lining the endocervical canal. The columnar epithelial cells in the junctional region are prone to metaplastic transformation in response to environmental stress and/or hormonal stimuli, which lead to the replacement of the columnar epithelium with stratified squamous epithelium.

As shown in Fig. 2 *A–C*, no detectable immunoreactivity for either Notch protein was observed in the normal columnar epithelium. However, the layer of reserve cells, which lie beneath the fully differentiated columnar epithelium and serve as its precursors, clearly expresses Notch (Fig. 2*C*). The areas where the epithelium becomes metaplastic exhibited strong staining with antibodies against both Notch proteins (Fig. 2 *A* and *B*). It should be noted that since it has been proposed that the reserve cells can give rise to squamous metaplasia, the staining of these cells shown in Fig. 2*C* may reflect expression in early metaplastic stages (28). We also note that the inflammatory cells consistently express Notch. The same results were

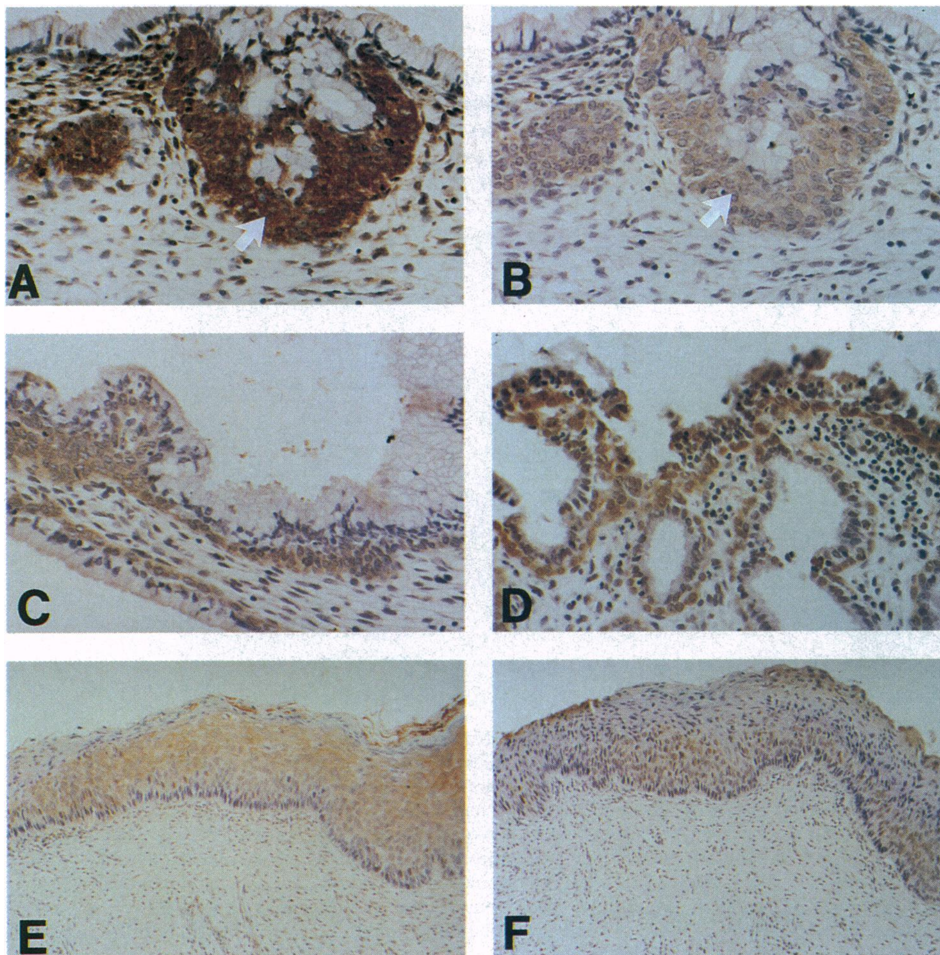

FIG. 2. Expression of Notch and TLE proteins in cervical tissues. (*A* and *B*) Expression of Notch 1 and Notch 2 in metaplastic tissue. Antibodies 15A (*A*) against Notch 1 and 6D (*B*) against Notch 2 were used as hybridoma culture supernatants at dilutions 1:20 and 1:5, respectively. No staining was observed in the columnar epithelium, while the metaplastic tissue (white arrow) gave strong cytoplasmic staining with both antibodies. (*C*) Expression of Notch proteins in the reserve cells. Staining with antibody 6D is shown. (*D*) Expression of TLE proteins in cervical metaplastic tissues. Sections were stained with antibody 4A that recognizes all four human TLE proteins (1:10 dilution). The nuclei of metaplastic and reserve cells were stained, while the columnar epithelium showed no staining. (*E*) Expression of Notch 2 in the squamous epithelium of the cervix. (*F*) Expression of TLE proteins in the squamous epithelium of the cervix. (*A–D*, ×280; *E* and *F*, ×112.)

obtained with other Notch 1-specific monoclonal antibodies as well as with the Notch 2-specific polyclonal PGHN serum, whereas cervical tissues treated with irrelevant antibodies showed no staining (data not shown).

Fig. 2*E* shows that Notch proteins were detected only in the stratum spinosum of the normal squamous cervical epithelium. This layer contains proliferating cells that are committed to the squamous fate. Notch expression was not detectable in the basal layer of undifferentiated cells or the keratinized, fully differentiated layer of cells that lies above the stratum spinosum (Fig. 2*E*). These results indicate that the expression of both Notch proteins is associated with the differentiation of columnar as well as squamous epithelia.

To determine whether similar expression patterns are exhibited by other genes that are also thought to be elements of the Notch pathway, we examined the expression of the TLE genes (18) in the same tissues. TLE expression in cervical tissues was monitored using monoclonal antibodies that recognize the entire TLE family (18). As shown in Fig. 2*D*, metaplastic epithelium and reserve cells expressed TLEs, while the normal columnar epithelium did not. As observed with Notch, the expression of TLEs in normal squamous epithelium was detected in the layer of committed proliferating cells (Fig. 2*F*). Thus in this case the expression of Notch and the TLE proteins coincides during normal and metaplastic cell-fate decisions. In agreement with the expression patterns in *Drosophila*, Notch activity in the human cervix is consistent with the notion that the pathway is not needed in terminally differentiated tissues but is associated with proliferative cell populations that are thought to be responsive to developmental signals (15).

Since cancerous cells are often considered to represent nonterminally differentiated cell populations, we also investigated Notch expression in neoplastic conditions of the cervix, which account for a significant percentage of human cancers (1). Using the available antibodies, we examined squamous carcinomas, which form the majority of cervical cancers, as well as the less common adenocarcinomas, which are thought to have a different pathogenesis (29). *In situ* and invasive squamous cell carcinomas were found to express Notch 1 (Fig. 3 *A* and *D*). When we monitored the expression of Notch 1 in cervical adenocarcinomas, we also detected high levels of expression in invasive (Fig. 3*B*) and *in situ* adenocarcinomas (results not shown). These results are in contrast to the absence of Notch expression in the differentiated columnar epithelial cells that these tumors have replaced (Fig. 2). Notch 2 expression paralleled Notch 1 expression in both carcinomas (data not shown). TLE expression was also elevated in adenocarcinomas (Fig. 3*C*), as well as in *in situ* squamous cell neoplasms (data not shown), but not in invasive squamous cell carcinomas (data not shown). These findings suggest that the modulation of Notch activity is not necessarily followed by the TLE expression profile.

The expression of Notch in the *in situ* squamous carcinoma depicted in Fig. 3*D* reveals (Fig. 3*D Inset*) the presence of Notch immunoreactive material in a subpopulation of the nuclei. Several previous studies have raised the possibility that Notch may participate directly in nuclear events by cleavage of the intracellular domain followed by translocation to the nucleus of the resulting fragment (7–9, 11). However, while artificially truncated forms of *Drosophila* Notch have been detected in the nucleus, extensive immunocytochemical studies failed to reveal nuclear Notch immunoreactive material during wild-type development (15). In contrast, recent immunocytochemical studies involving the expression of Notch in the rat retina revealed the existence of immunoreactive material that is recognized by Notch antibodies in the nuclei of a subpopulation of neurons (I. Ahmad, P.Z., and S.A.-T., unpublished data). The functional significance of nuclear Notch antigens is not clear, but it is possible that the cells expressing nuclear forms of Notch have a different developmental potential than the ones expressing Notch on the cell surface.

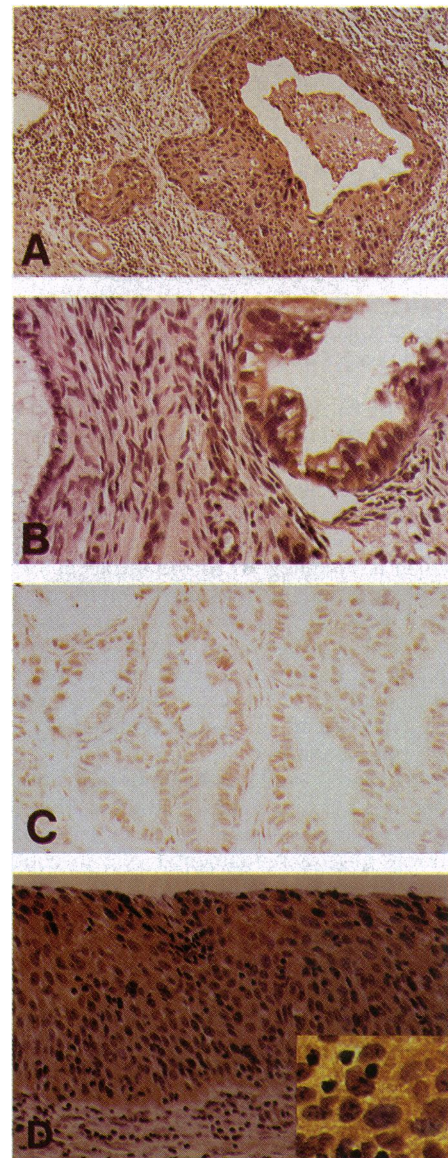

FIG. 3. Expression of Notch and TLE proteins in cervical squamous carcinomas and adenocarcinomas. Sections of invasive squamous carcinomas (*A*) and adenocarcinomas (*B*) showed expression of Notch 1 after staining with antibody 15A. The adjacent normal tissues as well as the stroma were not stained. (*C*) TLE staining of cervical adenocarcinomas using antibody 4A exhibited a similar pattern. (*D*) Staining of *in situ* squamous carcinoma with antibody 15A against Notch 1. (*Inset*) Magnified portion of the same tissue where nuclear staining is visible. (×260.)

Notch expression in cervical cancers raises some noteworthy possibilities regarding the potential role of Notch in neoplasias. Ellisen *et al.* (19) described chromosomal translocations that are associated with lymphoblastic leukemias. These chromosomal rearrangements predict truncated mutant Notch 1 proteins with most of the extracellular domain deleted. It has since been shown that similar deletions in transgenic flies, *Xenopus*, and mammalian cell lines cause gain-of-function—i.e. "activated"—phenotypes that appear to result from blocking the ability of precursor cells to respond to the proper developmental signals (5–11). In *Drosophila*, these cells were shown to differentiate eventually, when the expression of the mutant Notch subsides, even though they seem to acquire terminal fates different from their wild-type counterparts (8).

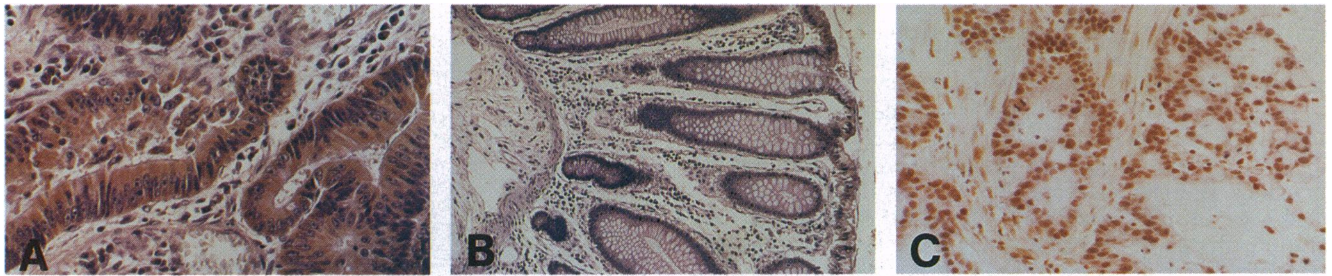

FIG. 4. Notch and TLE expression in colon adenocarcinomas. Sections of malignant (*A*) and normal colon (*B*) were stained for Notch 1. Expression of the protein was detected only in the malignant tissue (*A*) but not in normal glands (*B*). (*C*) Expression of TLE proteins was detected in the nuclei of malignant cells only. (×260.)

The results of Ellisen *et al.* (19), in conjunction with the analysis in *Drosophila*, indicate that Notch may act as an oncogene, at least during hematopoietic development. The involvement of abnormally expressed Notch-like proteins in epithelial neoplasias is also demonstrated by the development of tumors in mice, where the intracellular domain of the int-3 protein, which is homologous to the intracellular domain of Notch, is overexpressed (30). Given the general developmental role of the Notch pathway, one might expect that somatic mutations resulting in activation of the pathway in any uncommitted cell may prevent it from responding properly to normal developmental cues. If, for instance, a mutant cell is locked into a proliferative, undifferentiated state, this may cause a neoplastic condition.

In addition to the possible pathologies that a mutant Notch gene may induce, it is also important to consider the function that the normal Notch pathway may play in neoplasias. The Notch pathway plays a fundamental role in controlling the fates of undifferentiated, proliferative cell populations. As cells progress toward their terminally differentiated state, they may require the Notch pathway to be either "on" or "off" in order to progress to the next stage. The onset of a neoplastic condition is thought to involve the accumulation of mutations in uncommitted cells, so that they reach a point where the ability to respond properly to the available developmental signals is lost, and thus they fail to attain the normal differentiation state. Whether or not the Notch pathway is activated in a given malignancy will therefore depend on the developmental history and nature of the accumulated somatic mutations in these cells.

Based on this model, we expect that certain neoplasias will express Notch while others will not. We therefore examined the expression of both Notch and TLE proteins in colonic adenocarcinomas. As in cervical carcinomas, Notch 1 and TLE proteins were detected in these neoplasias (Fig. 4 *A* and *C*). The nonmalignant, normal glands showed no staining with either Notch 1 (Fig. 4*B*) or TLEs (not shown). Expression of Notch 2 paralleled that of Notch 1 exactly (not shown). Preliminary studies have also shown that Notch 1 is expressed in lung squamous cell carcinomas. This is in contrast to TLEs, which show no detectable expression in this kind of cancer (data not shown). This finding confirms that expression of the two proteins does not always coincide.

From the extensive developmental analysis in *Drosophila* we know that inactivation of Notch in cells that normally express it, or activation of Notch in nonterminally differentiated cells, results in derailing these cells from their normal differentiation course. It is therefore conceivable that agonists or antagonists of the Notch pathway may provide a means to manipulate the developmental fate of malignant cells, thus potentially offering a novel therapeutic approach.

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