Changing Roles of Cadherins and Catenins during Progression of Squamous Intraepithelial Lesions in the Uterine Cervix

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Uterine cervix represents a convenient model for the study of the gradual transformation of normal squamous epithelium via low- to high-grade squamous intraepithelial lesions (SILs). Because SIL, on the basis of the cytokeratins expressed, are thought to originate from the reserve cells, we analyzed whether SILs also show a reserve cell phenotype with respect to intercellular interactions. The changes in expression and subcellular localization of the components of the adherens junction and desmosomal complexes were investigated in normal, metaplastic, and premalignant cervical epithelium, as well as in cell cultures derived from these tissues. The results suggest that 1) during progression of SILs, E-cadherin is suppressed, with its role in cell-cell connections diminishing; 2) P-cadherin, in contrast, becomes the predominant cadherin in high-grade SILs; 3) the level of cellular α -catenin is dramatically decreased in high-grade SILs; 4) the level of β -catenin is decreased during progression of SILs, with plakoglobin suggestively becoming the predominant catenin mediating connection of cadherins to the cytoskeleton; 5) the assembly of desmosomes is affected during progression of SILs and is accompanied by a dramatically decreased expression for desmogleins and desmoplakins (I, II); and 6) expression of differentiation markers (involucrin, CK13) in high-grade SILs seems to be controlled by P-cadherin as opposed to E-cadherin in the normal tissue counterpart. We conclude that during development of cervical lesions substantial (both quantitative and qualitative) changes occur in cell-cell junctions, making the interactions of cells in lesions dissimilar from those of reserve cells, basal cells, or cells of immature squamous metaplasia, despite existing morphological similarity between all of these cell types and cells of high-grade lesions. (*Am J Pathol* 1999, 155:505–515)

Intercellular adhesions control normal tissue morphogenesis, including segregation of cell types, differentiation, and support of a particular tissue architecture.^{1–3} In epithelia, the central role in supporting normal cell and tissue morphology can be assigned to E-cadherin.⁴ E-cadherin participates not only in the interconnection of cells, but, together with the molecules comprising the adherens junction complex, is actively involved in signaling, in regulating the assembly of other junctions, and in controlling cell proliferation and motility.^{2, 5, 6} Disturbances in cell differentiation in epithelia observed during carcinogenesis correlate clearly with changes in intercellular adhesion.^{7, 8} E-cadherin, being in the center of cell-cell interactions, is usually affected during tumor progression and invasion in many types of epithelial tumors.^{4, 7} Affected E-cadherin-mediated adherens junctions result in changes in cell phenotype, increased cell invasiveness, increased cell motility, and other changes in vital cell functions.^{4, 7}

In tumors, however, one deals with the final result of a multistage selection process of various cell geno- and phenotypes. The final tumor cell population usually consists of a number of subpopulations that originate from consecutive changes during tumor progression, based on immunohistochemical and genetic studies.^{9, 10} It is of considerable interest to pinpoint the early changes in intercellular adhesion, which are associated with the development of hyperplastic and neoplastic cell phenotypes.

The multistage nature of carcinogenesis in cervical epithelium makes it possible to follow the events for a number of intermediate stages leading from a disturbance in proliferation and differentiation of squamous metaplasia, via low- and high-grade squamous intraepithelial lesions (SIL), to carcinoma.^{11, 12} SILs are thought to arise from the “transformation zone,” the squamocolumnar junction between ecto- and endocervical epithelium. Proliferation of the reserve cells and their differentiation via immature and mature squamous metaplasia into normal squamous epithelium serve the replacement of

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endocervical epithelium by ectocervical epithelium.^{12, 13} If the differentiation stimuli are blocked, these reserve cells may proliferate into an atypical immature squamous metaplastic epithelium or SIL.¹² Suggestively originating from the reserve cells, SILs are believed to inherit some differentiation features of their progenitors. SILs express mostly cytokeratins 6, 8, 17, and 18; in this they resemble reserve cell phenotype/differentiation, although they also express some keratins (5, 14, 19) common to both the reserve cells and the basal cells of normal squamous epithelium.¹⁴

In this study, we have analyzed immunohistochemically whether the intercellular adhesions typical for the reserve and basal cells are reproduced in SILs cells during the development and progression of these lesions. We have also analyzed biochemically the composition of intercellular junctions and whether the different phenotypes are controlled by different cadherins in primary cultures of cervical keratinocytes from normal tissue and lesions. Both immunohistochemical and cell culture experiments have demonstrated that abnormal proliferation in cervical tissue and altered cell differentiation are accompanied by substantial changes in junctional proteins with respect to the type of cadherins mediating cell adhesion, the composition of catenins in cadherin junctions, and the composition of desmosomes. Despite certain similarities of SIL cells with respect to the organization of adhesions observed in reserve cells, the major changes occur in SILs with respect to the number and composition of adherens junctions and desmosomes.

Materials and Methods

Tissue Samples

Tissue samples (biopsies and surgical specimens of lesions of uterine cervix) were collected from the archive and tissue bank at the Department of Pathology (Leiden University Medical Center). Tissues were routinely fixed in 4% formalin and embedded in paraffin. Sections, subsequent to those used for immunostaining, were stained with hematoxylin/eosin. A total of 30 specimens were analyzed, containing normal squamous epithelium and metaplastic epithelia (immature squamous metaplasia and mature squamous metaplasia), and low- and high-grade SILs. Because most of the sections also contained normal squamous epithelium and sometimes contained metaplasia along with SILs, the numbers for each tissue/lesion type were normal cervical epithelium, $n = 32$; immature squamous metaplasia, $n = 8$; mature squamous metaplasia, $n = 7$; low-grade SILs, $n = 8$, and high-grade SILs, $n = 9$. In addition, several cases of reserve cell hyperplasia (RCH) were studied ($n = 4$).

Antibodies

Antibodies against E-cadherin (HECD-1), P-cadherin (NCC-CAD-299), Ki-67 (MIB1), and cytokeratin 13 (1C7) were from Thamer Diagnostica BV (Uithoorn, the Netherlands). Monoclonal antibodies (mAbs) against E-cad-

herin (clone 36), P-cadherin (clone 56), α -catenin (clone 5), β -catenin (clone 14), γ -catenin/plakoglobin (clone 15), and pp120 (clone 98) were all from Transduction Laboratories (Lexington, KY). mAb against desmoplakins I+II (clone 115F) was kindly provided by Prof. D. Garrod (University of Manchester, Manchester, England). Anti-desmoglein antibody (clone DG3.10, recognizing desmoglein "band 3" polypeptide of the desmosomal complex) was from Progen (Heidelberg, Germany). Anti-involucrin mAb (clone SY5) was from Sigma Immunochemicals (St. Louis, MO). mAb aCAT-7A4 to α -catenin was from Zymed Laboratories (San Francisco, CA). mAb HECD-1 was used for both immunohistochemistry and blocking studies, mAb NCC-CAD-299 was used in blocking studies only, mAb anti- α -catenin (no. 5) was used for immunohistochemistry only. mAbs against E-cadherin (no. 36) and α -catenin (aCAT-7A4) were used for immunoblotting experiments.

Immunohistochemical Staining of Tissue Sections

From paraffin-embedded tissues 4- μ m sections were cut, mounted on glass pretreated with 2% 3-aminopropyltriethoxysilane (Sigma), and air-dried overnight at 37°C. Sections were deparaffinized in xylene, hydrated in a graded alcohol series, fixed in methanol (5 minutes room temperature, RT), incubated in 0.3% H₂O₂ in methanol (20 minutes), and subsequently washed with distilled water. To retrieve the antigen's reactivity, sections were pretreated before staining as described earlier.¹⁵ Briefly, sections were incubated for 10 minutes in boiling citrate buffer (0.01 mol/L citrate/0.01 mol/L Na-citrate, pH 6.0) or citrate-detergent buffer (0.01 mol/L citrate/0.01 mol/L Na-citrate, pH 6.0, 0.05% Dish Clean; Bosman Chemie, Heijningen, the Netherlands), cooled down in buffer for 2 hours, and subsequently incubated overnight at RT with the first antibody. Anti-involucrin mAb was used on non-treated sections. Binding of the mAb was detected using an anti-mouse ABC system (Dako, Glostrup, Denmark). Histology for all sections was analyzed by two pathologists independently (H.v.K., G.J.F.). Staining within the lesion, where possible, was related to the staining of normal tissue (ecto- and endocervical epithelium) within the same section.

Cells and Culture

For biochemical analysis of the cell-cell junctions in cervical keratinocytes we used primary cultures of cells from tissue samples of normal and abnormal cervical tissues, isolated as previously described (Van Dorst et al, manuscript submitted for publication, and Ref. 16). Cells were cultured until 80% confluence in keratinocyte-SFM culture medium supplemented with human recombinant epidermal growth factor and bovine pituitary extract (Gibco BRL, Paisley, Scotland). Before the experiments, medium was replaced for 24 hours with Dulbecco's minimum essential medium (DMEM)/HAM's F12 1:1 (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum. Cadherin-mediated adhesions were disrupted by

culturing the keratinocytes in the presence of function-blocking antibodies for E-cadherin (HECD-1, IgG1) and P-cadherin (NCC-CAD-299, IgG1) at a concentration of 20 $\mu\text{g/ml}$ for 24 hours in DMEM/HAM's F12 medium. Three cell cultures/lines representing normal cervical keratinocytes and two different stages of SIL development were used. Normal cervical keratinocytes (NCKs) were expanded immediately after isolation to yield a large quantity of cells (= passage 1), which were frozen in liquid nitrogen. For experiments the NCK cells were thawed and cultured for one extra passage (= passage 2). Two established cell cultures, one representing a low-grade SIL (612) and one a high-grade SIL (612.6E) were derived from histologically characterized biopsy specimens, as was previously described (Van Dorst et al, manuscript submitted for publication). All three cell types were able to reproduce the morphology of the respective tissue of origin in the presence of calcium in culture medium, as described (Van Dorst et al, manuscript submitted for publication).

Immunoprecipitation and Immunoblotting

Cross-linking of the molecules of the cadherin-catenin complex was performed after the method of Hinck et al,¹⁷ using dithio-(bis)-succinimidylpropionate (DSP), a cleavable bifunctional linker (Pierce Chemical Co., Rockford, IL). Detergent extraction of cells (1% Triton X100 or 25 mmol/L CHAPS ([3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate])),¹⁸ nonextracted cell fraction in 1% sodium dodecyl sulfate, immunoprecipitation, and immunoblotting were as previously described.^{19, 20} In all experiments, an equal amount of protein was analyzed per cell type.

Results

In this study the following criteria were used to define cells as being abnormally differentiated and comprising a SIL: 1) morphology, ie, abnormal maturation, nuclear enlargement and atypia characterized by pleomorphism, coarse chromatin clumping, and irregular contours; loss of polarity; frequency and level of mitosis^{12, 21}; 2) expression of Ki-67, a marker that discriminates between cells in G₀ phase and the cycling cell population, and which has been used previously as a marker in the classification of cervical lesions²²; and 3) expression of involucrin, a marker for terminal differentiation of keratinocytes,²³ particularly cervical keratinocytes.²² The immunohistochemical results presented are grouped in the figures according to the type of tissue or lesion and not by a particular marker investigated, to allow a comparison of the patterns for different markers on consecutive sections.

E-Cadherin

In normal endocervical (glandular) epithelium E-cadherin was present at the lateral domains of cells, with no detectable intracellular presence, in contrast to reserve

Figure 1. Expression of E- and P-cadherins in endocervical (columnar) (top) and reserve cells (bottom) cells. E-cadherin is present in columnar cells, but is absent from the reserve cells. Reserve cells express P-cadherin, which is absent from columnar cells (two top panels). In case of reserve cell hyperplasia (two bottom panels), hyperplastic cells (arrow) express both types of cadherin. E, E-cadherin; P, P-cadherin. Bar, 50 μm .

cells, which were negative (Figure 1, top). Expression of E-cadherin in reserve cells was noted only in relation to the development of a reserve cell metaplasia (Figure 1, bottom). Actively proliferating cells of immature squamous metaplasia were marked by a dramatic increase in intracellular staining for E-cadherin (as compared to glandular epithelium), although the presence of a large amount of E-cadherin at cell-cell boundaries was still clearly evident (Figure 2). A slight increase in intercellular E-cadherin was observed in areas where immature squamous metaplasia cells undergo maturation, as shown by involucrin expression. No substantial differences were found with respect to E-cadherin expression and localization in mature squamous metaplasia (not shown), as compared to normal squamous epithelium (Figure 3). A decreased presence of E-cadherin at cell-cell boundaries was noted for cells of the basal layer of normal squamous epithelium, with a subsequent increase in parabasal and, especially, suprabasal layers (Figure 3). It should be noted that in lower layers of normal squamous epithelium, in addition to lateral staining, a substan-

Figure 2. Expression of the adherens junction-associated molecules in immature squamous metaplasia. Here and later: Ki, Ki-67; I, involucrin; E, E-cadherin; P, P-cadherin; α , α -catenin; β , β -catenin; γ , plakoglobin; 120, pp120. Note the dramatically increased total level of P-cadherin (P) and plakoglobin (γ) and the increased cytoplasmic staining for E-cadherin (E) and pp120 (120). Note also a relatively increased level of all three catenins (α , β , γ) at the cell-cell boundaries. Ki-67 and involucrin allow the estimation of the level of cell proliferation and terminal differentiation in a particular area.

tial intracellular staining for E-cadherin was observed. This intracellular staining was practically absent from columnar epithelium (Figure 1, top) and most likely reflects active rearrangement of adherens junctions in lower layers of normal squamous epithelium.

No substantial decrease in E-cadherin expression was observed in low-grade SILs. However, a relatively increased intracellular staining was noticeable in proliferating layers of cells in the lesions. To some extent, in the majority of low-grade SILs, the level of E-cadherin expression and its localization in several basal layers reproduced the basal layer of normal squamous epithelium (Figure 3).

Two SILs (2/8) showed a dramatic decrease in E-cadherin in the basal/abnormally differentiated layers, with the molecule reappearing in the upper, more differentiated layers, practically at the same levels as in other low-grade SILs (Figure 4). These latter lesions were difficult to place strictly in either the low- or high-grade SIL category. According to morphology and degree of proliferation, as assessed by Ki-67 expression and the frequency of mitosis, they were grouped as low-grade SIL, but changes observed in the cadherins and catenins affected approximately half of the thickness of the squamous tissue (not shown).

In high-grade SILs, a strong decrease in E-cadherin expression was noticed. In some lesions an almost complete loss of E-cadherin was found in the basal layer, with expression being partially restored in suprabasal layers. In the majority of high-grade SILs showing active proliferation through the entire thickness of the epithelial layer and a complete loss of involucrin expression, a clear decrease in E-cadherin expression was found in all cells. The presence of E-cadherin at the cell-cell boundaries

Figure 3. Expression of adherens junction molecules in normal cervical epithelium (NSE) and low-grade (LG-SIL) and high-grade (HG-SIL) SILs. Abbreviations are as in Figure 2. Note the relocation of E-cadherin (E) and α - and β -catenin from the membrane to the cytoplasm in high-grade SILs. P-cadherin (P), plakoglobin (γ), and pp120 (120), in contrast, are present at the cell membrane and at higher levels.

Figure 4. A particular case of low-grade SIL, showing loss of expression of α - and β -catenin. Abbreviations are as in Figure 2.

could be found only in the very upper SIL layers, and in the majority of cells only a moderate intracellular staining was present (Figure 3).

P-Cadherin

P-cadherin was practically absent from endocervical (simple) epithelium, in contrast to reserve cells, which were positive for P-cadherin (Figure 1). We also noted that in areas of an enriched presence of reserve cells, the upper layers of glandular epithelium expressed low levels of P-cadherin, which disappeared in glandular epithelium that was just slightly distant from this site (not shown). Cells of reserve cell hyperplasia retained expression of P-cadherin (Figure 1) along with *de novo* expressed E-cadherin.

Development of immature squamous metaplasia was accompanied by a dramatic increase in P-cadherin expression, present both at the cell-cell boundaries and in the cytoplasm (Figure 2). During maturation of immature squamous metaplasia, the expression of P-cadherin remained, being present in morphologically normal (similar to normal cervical epithelium) basal, parabasal, and suprabasal cells of mature squamous metaplasia (not shown). Only further maturation of mature squamous metaplasia to normal cervical epithelium resulted in the disappearance of P-cadherin from all cell layers except for the basal layer (not shown). In normal cervical epithelium, P-cadherin was present only in cells of the basal layer, with some traces of expression in parabasal cells (Figure 3), in accordance with patterns observed for normal skin keratinocytes induced to differentiate,²⁴ and in other squamous epithelia.²⁵

In low-grade SILs, an up-regulation of P-cadherin was observed in intermediate and upper layers (not shown). Staining was predominantly located at the cell-cell boundaries.

In high-grade SILs, P-cadherin was slightly decreased in comparison to cells of immature squamous metaplasia and even low-grade SILs. However, in contrast to the pattern observed for E-cadherin, P-cadherin was present at cell-cell boundaries, with its expression increasing from the lower to the upper layers of high-grade SIL tissue (Figure 3). These observations suggest that P-cadherin actively participates in cell-cell adhesion in SIL lesions, especially in those of a high grade.

α -Catenin

Expression of α -catenin, a molecule that mediates interactions of classic cadherins with the actin cytoskeleton,^{26, 27} was high in immature squamous metaplasia (Figure 2), during proliferation into mature squamous metaplasia (not shown), as well as in normal cervical epithelium (Figure 3).

In low-grade SILs, no dramatic decrease in α -catenin expression was observed. In general, at the levels of expression and subcellular localization of α -catenin (at cell-cell boundaries, but also slightly in the cytoplasm), as observed in basal layer cells, most low-grade SILs resembled basal cells of normal cervical epithelium (Figure 3). In two low-grade SILs (with grossly disturbed E-cadherin expression), a very strong decrease in α -catenin staining was observed in the lower basal layers (Figure 4). Remarkably, no reexpression of α -catenin in the upper layers of these lesions, as was seen for E-cadherin, was observed.

For high-grade SILs, a strong reduction of α -catenin expression was observed in the majority (8/9) of the lesions (Figure 3). Only in the very upper layers could the presence of some α -catenin molecules be detected at cell-cell boundaries.

β -Catenin and Plakoglobin (γ -Catenin)

Both β -catenin and plakoglobin can participate in the formation of the cadherin connection to α -catenin,^{26, 28} whereas plakoglobin is additionally involved in desmosome formation by the mediation of the connection of desmoglein to desmoplakins.^{29, 30} As compared to normal endocervical epithelium, no quantitative change and/or relocation of β -catenin was observed during the development of immature squamous metaplasia (Figure 2). For plakoglobin, however, an increased expression was observed in immature squamous metaplasia cells, including enhanced cytoplasmic staining (Figure 2). Mature squamous metaplasia showed a high level of plakoglobin, both at the cell membranes and in the cytoplasm of less differentiated cells (not shown). No decrease in or relocation of either β -catenin or plakoglobin was obvious during maturation of mature squamous metaplasia into normal cervical epithelium (Figure 3).

In low-grade SILs both β -catenin and plakoglobin were present, and the level of expression, with an enhanced

cytoplasmic staining in basal layers cells, was similar to expression observed in basal layer cells of normal cervical epithelium (Figure 3). In one SIL, a practically complete reduction of β -catenin expression was observed, accompanied by a strong reduction in E-cadherin and α -catenin, but no changes in plakoglobin expression levels were observed (Figure 4). The other case with a reduction of E-cadherin and α -catenin described earlier, expressed levels of β -catenin similar to those observed in other low-grade SILs.

In high-grade SILs a relative reduction in the level of β -catenin expression was observed (9/9), whereas the presence of β -catenin at cell-cell boundaries was also decreased (Figure 3), as has also been observed for various other types of epithelial tumors.³¹ Some reduction of plakoglobin expression was seen in the majority of high-grade SILs (6/9), but not as much as was observed for β -catenin (Figure 3).

pp120

pp120, a catenin-like molecule, is a tyrosine kinase substrate implicated in receptor-ligand-induced signaling by growth factors and in transformation by *src*.³² pp120 colocalizes with E-, P-, or N-cadherin³² and binds to the juxtamembrane domain of the E-cadherin cytoplasmic tail,³³ not competing for binding with β -catenin or plakoglobin.

In normal endocervical epithelium, pp120 was present at the lateral domains of cells, with no pronounced intracellular presence (not shown). In immature squamous metaplasia, both a high level of membranous as well as cytoplasmic staining was observed (Figure 2), suggesting a certain increase during cell proliferation in metaplasia. In mature squamous metaplasia a strong intercellular staining was observed (not shown) that was similar to normal cervical epithelium (Figure 3).

In low-grade SILs, a slight increase in expression was observed for pp120 that was also seen in high-grade SILs (Figures 3). In all cases of SIL, a normal membranous staining with no increased cytoplasmic staining was observed, as was observed for other catenins, as far as can be judged by immunohistochemistry.

Desmosomes (Desmocollins and Desmogleins)

Desmosomes provide the cells with binding domains for intermediate filaments of the cytokeratin network and are thus required for tissue organization.³⁴ Desmosomes are formed by two different types of transmembrane proteins belonging to the cadherin family of proteins, desmogleins and desmocollins, associating with various cytoplasmic proteins, like desmoplakins and plakoglobin, and some other molecules.³⁵ Three of the desmosomal proteins, desmocollin, desmoglein, and desmoplakin, show different protein forms, either derived from alternative splicing or coded by different genes, which are either tissue specifically or ubiquitously expressed.³⁶

In immature squamous metaplasia, an increased intracellular staining for desmoplakin and an absolute ab-

Figure 5. Desmosomes in the progression of SILs. Shown are immature squamous metaplasia (ISM), normal cervical epithelium (NSE), and a case of high-grade SIL (HG-SIL). Note the absence of desmoglein in high-grade SIL as also seen in reserve cells, basal cells of normal squamous epithelium, and cells of immature squamous metaplasia. Desmoplakins, however, are well expressed in cells of immature squamous metaplasia and basal cells of normal cervical epithelium, but are dramatically decreased in a high-grade SIL.

sence of desmoglein were observed (Figure 5). During maturation of mature squamous metaplasia (not shown) into normal cervical epithelium, formation of desmosomes resembled the pattern observed in normal cervical epithelium. Both desmoglein as well as desmoplakins (I and II) showed hardly any expression in the basal layer of normal cervical epithelium, whereas both suprabasal cells and differentiating cell layers showed a membranous staining, except for the superficial layers, which were largely negative (Figure 5).

In low-grade SIL lesions a dramatic decrease in the expression of desmoglein, and, especially, desmoplakins was already observed as compared to normal cervical epithelium (not shown). In high-grade SIL lesions, the low expression levels of both desmoglein and desmoplakins remained (Figure 5). A punctate staining for desmoplakins as observed in some high-grade lesions probably suggests the presence of "half-desmosomes" on the cell surface. All changes were consistently present in all SILs analyzed.

Biochemical Analysis of the Composition of Cell-Cell Junctions in Established Cell Cultures Representing Various Stages of Cervical Lesion Development

Changes in cadherin and catenin expression patterns during SIL development and progression suggest that the roles of E- and P-cadherin, as well as their involvement in adherens junction formation, are changing. The results also suggest that the composition of cadherin adhesion complexes may differ greatly between normal and neoplastic keratinocytes. To analyze biochemically the components of cell-cell junctions present in cervical keratinocytes, we used three different cell types: NCKs and two cell cultures, one representing a low-grade (612) and one a high-grade (612.6E) SIL. The latter two were derived from biopsy specimens from patients as previ-

Figure 6. Biochemical analysis of adherens junction proteins in normal cervical keratinocytes (NCKs) and cultured cells from low-grade (612) and high-grade (612.6E) lesions. Cells were cultured in keratinocyte medium and before analysis were transferred into DMEM/HAM's F12 medium to induce differentiation. **A and B:** Cytokeratin 13, involucrin, E-cadherin, catenins, and pp120 in total cell lysates. **C:** Detergent (25 mmol/L CHAPS) solubility of catenins in cells. Note that the solubility of α - and β -catenin is increasing from normal to high-grade lesion cells. At the same time the soluble fraction of plakoglobin remains the same, although its total amount is increasing; thus the insoluble plakoglobin is increasing. **D:** Detergent-soluble (25 mmol/L CHAPS) and detergent-insoluble P-cadherin. Note that the relative ratio of insoluble to soluble P-cadherin increases from NCK to 612.6E cells. **E:** Immunoblotting with antibodies to E-cadherin and catenins on immunoprecipitates prepared using anti-E-cadherin mAb. Before lysis, the proteins of cadherin complex were cross-linked using the cleavable linker dithio-(bis)-succinimidylpropionate (DSP). Note decreasing amounts of catenins coprecipitated with the same amount of E-cadherin.

ously described (Van Dorst et al, manuscript submitted for publication).

To analyze whether culturing the cells did induce squamous/terminal differentiation to the degree observed *in situ*, total cell lysates of cells cultured in the presence of normal (1.8 mmol/L) Ca^{2+} concentration were probed for involucrin and cytokeratin 13 (Figure 6A). Both markers were expressed at the highest level in NCKs, at a decreased level in 612, and at an even lower level in 612.6E cells, confirming that, at least with respect to expression of these differentiation markers, cells were similar to the lesions *in situ*. In another study we have also analyzed the expression pattern of other cytokeratin chains in these cells and concluded from the expression of both squamous-specific and differentiation-specific cytokeratins that the various cells resembled the respective tissues *in situ* (Van Dorst et al, manuscript submitted for publication).

The relative expression levels of the adherens junction-associated proteins, either cadherins (E- and P-cadherin) or catenins (α -catenin, β -catenin, pp120), did not show any remarkable differences between the cell lines when total cell lysates were analyzed (Figure 6B), with the exception of plakoglobin, that was clearly increased in 612.6E cells.

Detergent extractability (25 mmol/L CHAPS) analysis showed that with an increasing grade of SIL, more and more α -catenin, β -catenin (Figure 6C), E-cadherin, and pp120 (not shown) was detectable in the soluble fraction. In contrast, both P-cadherin (Figure 6D) and plakoglobin (Figure 6C) showed a decreased solubility with increasing grade of SIL. This suggested that an increasing fraction of P-cadherin was involved in mediating cell-cell adhesions in high-grade SIL cells, which was possibly connected to the actin cytoskeleton via plakoglobin, which also showed a decreased solubility (Figure 6C). In contrast to some observations published earlier,^{37, 38} extraction with Triton X100 extracted all cadherins from cervical keratinocytes. The pellets obtained did not contain traces of E-cadherin, as was tested in immunoblotting (not shown).

To investigate the association of E-cadherin molecules with catenins in these SIL-derived cells, the adherens junction complexes were cross-linked by the use of a bifunctional reagent (as earlier described by Hinck et al).¹⁷ Immunoprecipitated E-cadherin complexes were analyzed for the presence of catenins in immunoblotting (Figure 6E). In E-cadherin immunoprecipitates a tremendous decrease was detected for α -catenin, β -catenin, and plakoglobin associated with E-cadherin, increasing with grade of SIL. Small differences between results obtained with the cultured cells and our immunohistochemical data may be related to the short time of the differentiation.

Regulatory Roles of Cadherins in the Expression of the Markers for Terminal and Squamous Differentiation

To further analyze the roles of the cadherin junctions in cultured SIL cells, function-blocking antibodies for E-cadherin and P-cadherin were used. Cells were incubated for 24 hours in the absence or presence of function-blocking antibodies, after which cell lysates were analyzed for expression of the terminal differentiation marker involucrin.

Morphologically, the strongest effects were seen when the cells from both low- and high-grade SILs were cultured in the presence of anti-E-cadherin mAb or the mix of anti-E- and P-cadherin antibodies (Figure 7). For the mix, the effect on cell morphology was more pronounced than that for either antibody alone. Cells no longer formed tight cell-cell adhesions, as observed during culturing without antibodies. Instead, loose clumps of cells were seen, floating on top of each other, with hardly any cell-cell contact. The effect of antibodies on low-grade SIL cells (612) was more pronounced than on high-grade SIL cells (612.6E).

In contrast to morphology changes, the roles of E- and P-cadherins in regulating the expression of involucrin were totally different in low- and high-grade SIL cells. Thus, in 612 cells the expression of involucrin was independent of the formation of cadherin junctions. In high-grade SIL-derived cells, 612.6E addition of the mAb to P-cadherin, but not to E-cadherin, blocked the expres-

Figure 7. Analysis of the presence of cadherin-function blocking antibodies on cultured cells from low-grade (612) and high-grade (612.E6) lesions. Cells were either cultured in keratinocyte medium (K) or DMEM/HAM's F12 medium (D) in the absence or presence of E- and P-cadherin function blocking antibodies. **Top:** Morphological effects on cell growth. **Bottom:** Involucrin expression in total cell lysates. Note the decrease in involucrin expression in high-grade cells in the presence of P-cadherin antibodies. E, Anti-E-cadherin antibody; P, anti-P-cadherin antibody; E+P, anti E- and P-cadherin antibodies.

sion of involucrin. It should be noted that a mix of anti-cadherin mAbs has a slightly stronger effect than anti-P-cadherin antibody alone. This observation supports the increased role for P-cadherin in the regulation of high-grade SIL cells.

Discussion

Squamous intraepithelial lesions are viewed as precursors of cervical carcinoma, although it may take decades before carcinoma actually develops, and even high-degree atypias may regress in a large percentage of cases.¹¹ SILs are thought to arise from the "transformation zone" (squamocolumnar junction) because of a disturbed proliferation of the reserve cells.^{12, 13} Suggestively originating from the reserve cells, SILs are believed to inherit some differentiation features of their progenitors. To some extent this has been demonstrated by studies of cytokeratin expression, where reexpression of the reserve cell cytokeratins was demonstrated for SIL cells.¹⁴

Cadherins, the transmembrane components of adherens junctions, mediate via homotypic interactions and binding to the cytoplasmic catenin molecules the interaction with the actin cytoskeleton, and in this way they play an important role as morphoregulatory molecules.^{2, 4, 39, 40} In squamous epithelia, E-cadherin is one of the major cell adhesion molecules defining the architecture and differentiation of keratinocytes.^{41, 42} Evidence is accumulating that E-cadherin may perform as a tumor-suppressor and invasion-suppressor molecule^{7, 8} and is functionally disturbed during carcinogenesis of various epithelial tissues, including cervical lesions and carcinomas.^{43, 44} In addition to changes in cell-adhesion molecules like cadherins,⁴³⁻⁴⁵ changes in catenins^{31, 46} have been observed in squamous epithelium, but not in great detail for cervical epithelium.

In this study we compared the expression pattern and organization of various molecules comprising the adher-

ens junctions and desmosomes, as expressed in normal cervical epithelium, during transdifferentiation and in SIL development and progression. In this way we wanted to establish whether SILs are derived from reserve cells, and therefore whether a reserve cell phenotype (with respect to intercellular adhesion) is reproduced in SILs, similar to that reported for cytokeratins.¹⁴

To some extent the adherens junctions of SILs do reproduce intercellular junctions of the reserve cell. A clear decrease in E-cadherin and an increase in P-cadherin observed during SIL progression may be viewed as a turn to the reserve cell phenotype, with a prevalence of P-cadherin. The basal layers of cells of low-grade SILs reproduce, in general, the type of adherens junctions and desmosomes that are found in basal cells of normal squamous epithelium or in immature squamous metaplasia. However, the expression and functionality of the individual molecules comprising the adherens junctions and desmosomes in SILs differed quite substantially from the junctions of normal progenitor cells. The major differences were a nearly complete disappearance of α -catenin in SIL cells, along with a decrease in β -catenin and an increase (!) in plakoglobin. Among desmosomal proteins, not only was desmoglein low in SILs, similar to the cells of immature squamous metaplasia, but so was desmoplakins. Most of the lesions showed a uniform pattern of changes. The immunohistochemical results were all confirmed at the biochemical level, using cell cultures of normal cervical keratinocytes and SIL cells.

During SIL progression the role of E-cadherin in cell-cell adhesion diminishes, and the molecule is practically absent in high-grade SILs. The impression is that the cells switch to another type of functional adherens junction, ie, P-cadherin, which seems to employ mainly plakoglobin instead of β -catenin (see Figures 3 and 6) as the connector to the actin cytoskeleton complex. Determination of whether the observed down-regulation of either E-cadherin or the catenins, via functional mutations or at the transcriptional level, results in down-regulation/inactivation of the other, as has been shown *in vitro*,^{38, 47} needs further investigation. However, observations in this study suggest that inasmuch as the existing coordination between E- and P-cadherin is disturbed only in high-grade SILs, down-regulation of E-cadherin must be at the transcriptional level.

A switch of E-cadherin to P-cadherin adhesions during SIL progression might explain the lack of terminal and squamous differentiation in SILs. Both cadherins are partially redundant in epidermal stratification in skin.⁴⁸ Basal cells, which express both cadherins, could use either cadherin to induce stratification; however, superficial cells are dependent on E-cadherin for further stratification.⁴⁸ In high-grade SIL, the expression of P-cadherin was observed throughout the lesion, suggesting that squamous and terminal differentiation cannot take place because of the presence of P-cadherin. In squamous carcinoma cell lines an expression pattern similar to that in the cervical lesions studied was observed. Expression levels of E- and P-cadherin were about the same in normal squamous keratinocytes, whereas reduced E-cadherin and high P-cadherin expression was ob-

served in squamous carcinoma cell lines.⁴⁹ This might indicate that upon malignant transformation of cells, expression of P-cadherin inhibits or induces specific signals, resulting in a lack of squamous and terminal differentiation, as indicated by the lack of CK13 and involucrin expression.

The most remarkable observation in SIL cells is the nearly complete absence of α -catenin and its decreased association with the E-cadherin/ β -catenin complex. Normally, α -catenin allows the cadherin molecules to anchor to F-actin filaments. It is not absolutely clear whether the reduced amount of cellular α -catenin is still sufficient to provide the anchor for P-cadherin adhesions as observed in this study, or whether another molecule (ie, vinculin, relatively homologues to α -catenin)^{50, 51} takes its place. The latter possibility was shown for some tumor cells by Hazan et al,³⁷ and more recently it was shown that vinculin can form a complex with α -catenin and thereby participate in the formation of adherens junctions.⁵² However, we did not find any evidence for this in our *in vitro* cultures, because no vinculin was coimmunoprecipitated with E-cadherin (data not shown). Whether the absence of α -catenin was caused by inactivating mutations in SIL cells remains to be investigated. However, the decrease in this catenin observed for all high-grade SILs suggests that regulatory mechanisms are responsible for this. Our observations are substantiated by the fact that α -catenin is also decreased in some other types of carcinomas.⁴⁶

During the progression of cervical lesions, we observe an increase in plakoglobin expression. It is plausible that overexpression of plakoglobin in SIL cells may lead to substantial changes in the composition of adherens junctions, switching from E-cadherin/ β -catenin/ α -catenin to P-cadherin/plakoglobin complexes. Forced overexpression of plakoglobin was shown to induce a decrease in cadherin-complexed β -catenin, probably regulated via the transcriptional pathway with degradation of noncomplexed extrajunctional β -catenin.⁵³ Furthermore, both β -catenin and plakoglobin are capable of binding LEF-1, a transcription factor, and participate in the transcriptional activation of other genes.

The role of pp120, shown to be highly expressed in both normal and SIL cells, remains obscure. It has been shown that pp120 can form complexes with the cadherin-catenin cell adhesion complexes and is necessary for both adhesion formation and lateral clustering,³³ but its complete function in cell-cell adhesion and signaling is not yet known.

The assembly of desmosomes in all SILs was grossly affected, showing a strong decrease in expression of both desmoplakins and desmoglein, similar to observations in squamous cell carcinomas.^{36, 54} A decrease in both desmoplakins and desmoglein in oral squamous cell carcinomas was correlated with poor differentiation, invasiveness, and lymph node metastases.⁵⁵ Clearly, by itself the disturbance in desmosomal plaques does not lead to invasive properties of cells, because in the case of SILs, it was observed for noninvasive precancerous lesions. The assembly of desmosomes is believed to be

dependent on E-cadherin and proper adherens junction formation⁵⁶⁻⁵⁸ and an intact actin and cytokeratin network.⁵⁷ Down-regulation of E-cadherin was shown to result in a reduction of desmosome formation and normal stratification.^{56, 59} Apparently, P-cadherin cannot take over the function of E-cadherin in desmosome formation.⁴⁸ Furthermore, desmosome formation is largely dependent on free plakoglobin.^{56, 60, 61} During SIL progression, we observed a relative decrease in the level of free plakoglobin (Figure 6D). In the studied cervical lesions we thus observe changes in all of the relevant molecules necessary for proper desmosome formation, ie, improper formation of adherens junctions using P-cadherin instead of E-cadherin, a large pool of cytoplasmic α - and β -catenins, occupied plakoglobin, as well as an impaired cytokeratin network (Van Dorst et al, manuscript submitted for publication). All of these changes contribute to the lack of desmosome formation during SIL progression.

Various mechanisms, probably all working together, are possibly involved in SIL development. A genetic mechanism, regulating the expression of either E-cadherin or P-cadherin during normal cervical differentiation, might be disturbed during SIL progression, like mutations leading to gene inactivation or promotor silencing. Because P-cadherin can still form adherens junctions but is not able to induce squamous/terminal differentiation, this might lead to uncontrolled proliferation of cells.⁴⁸ We previously reported that overexpression of Ep-CAM in cells interconnected by classic cadherins leads to abrogation of adherens-type junctions and a reduction in the total cellular α -catenin.¹⁹ In our previous study²² we showed Ep-CAM overexpression during SIL progression, and in this study we observe a down-regulation of α -catenin. Both phenomena possibly result in a down-regulation and/or abrogation of E-cadherin-mediated adherens junctions. Another option might be the involvement of oncoproteins. It was demonstrated that the retinoblastoma protein was able to induce E-cadherin expression.⁶² In nearly all cases of SIL, human papilloma virus (HPV) is involved, and HPV protein E7 is able to inactivate the retinoblastoma protein. This could thus lead to a decreased activation of E-cadherin expression. Because the overall impression is that genetic inactivation of E-cadherin, as observed for some tumors,¹⁵ does not take place during SIL progression, the suppression of E-cadherin rather takes place at the regulatory level, probably via inactivation of the retinoblastoma protein.

In conclusion, cell adhesion complexes are dramatically changed, especially in high-grade SIL, as compared to both normal squamous epithelium and reserve cells, which are both proliferating and undergoing transdifferentiation. Despite the fact that a rather organized cell structure is formed during the onset of cervical lesions, great disturbances in cell-cell interactions are present. For pathological purposes, both the expression of P-cadherin and the absence of α -catenin and desmoplakin expression throughout the lesion can be used in the classification of SIL.

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