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Carcinomas of Bartholin's Gland

Histogenesis and the Etiological Role of Human Papillomavirus

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In this study, we examine 10 primary carcinomas of Bartholin's gland, including seven squamous carcinomas, two adenoid cystic carcinomas, and one adenocarcinoma, as well as four non-neoplastic Bartholin's gland. Six of seven squamous cell carcinomas contained human papillomavirus (HPV) type 16 DNA detectable by the polymerase chain reaction; one of these demonstrated HPV type 16 by* in situ *hybridization. The two adenoid cystic carcinomas, the adenocarcinoma, and the non-neoplastic Bartholin's gland epithelium showed no evidence of HPV DNA by polymerase chain reaction or* in situ *hybridization. A panel of eight antibodies (Cam 5.2, B72.3, CEA, EMA, MCA, Lewis X, ER, and PR) demonstrate that the squamous, transition zone, duct, acinar, and myoepithelial cells of Bartholin's gland are antigenically distinct, and are similar to those reported in analogous areas of the uterine cervix. Squamous carcinoma and adenocarcinomas of Bartholin's gland are antigenically similar, and seem to arise from the transition zone of the Bartholin's gland duct. The origin of adenoid cystic carcinomas is more difficult to determine; it is distinct from squamous and adenocarcinomas and seems more likely to arise from myoepithelial cells. We conclude that adenocarcinoma and squamous cell carcinoma of Bartholin's gland arise in the transition zone of Bartholin's gland, which is similar to the transition zone of the uterine cervix. We also show that HPV is associated with Bartholin's gland carcinoma and may play a role in the genesis of malignancy. (Am J Pathol 1993, 142:925–933)

Carcinomas of Bartholin's gland are rare tumors that account for less than 1% of all gynecological malignancies.[1] Two major histological types, squamous cell carcinoma and adenocarcinoma, account for 80% to 90% of primary cases. The remainder are adenoid-cystic, transitional, or undifferentiated carcinomas. Initial observations relating Bartholin's gland carcinomas to a previous history of Bartholin's duct cysts suggested an inflammatory genesis to these tumors.[2] However, subsequent studies identified a history of preceeding vulvar cysts or inflammation in less than 10% of cases.[3-5] Podratz et al noted that that 27% of patients with vulvar carcinomas including Bartholin's gland carcinomas had a second primary, 58% of which arose in hormone-sensitive tissues.[6] Second primaries have been described in the endometrium,[7] cervix,[8,9] breast,[9] and ovary.[10] These associations suggested a possible etiological role of hormones in these tumors. Recent studies have confirmed the presence of estrogen and progesterone receptors in glandular carcinomas of Bartholin's gland,[11] yet, to our knowledge, this has not been the case with squamous cell or adenoid cystic carcinomas.[12]

Trelford noted that three of five patients with Bartholin's gland carcinomas had a concurrent primary squamous cell neoplasm of either the cervix or vagina and suggested that these Bartholin's gland carcinomas might be included in the group of genital tract tumors occuring at squamo-columnar junctions.[13] Harer also noted, in 1933, that as with cervical cancer, no cases of Bartholin's gland carcinoma had been reported in virgins.[2]

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The predominance of squamous cell carcinomas and adenocarcinomas in Bartholin's gland parallels the histological types of carcinomas found in the uterine cervix. We believe that these associations are more than coincidental. In this study, we examined the antigenic phenotype of 10 Bartholin's gland carcinomas and four non-neoplastic Bartholin's glands by immunohistochemistry in an attempt to localize the microanatomic site of malignant transformation. In addition, we examined these tumors for the presence of human papillomavirus (HPV) sequences by *in situ* DNA hybridization and the polymerase chain reaction (PCR) to investigate a possible etiological role of HPV infections in the genesis of Bartholin's gland carcinomas.

Materials and Methods

Case Selection

Cases were selected from the archives of the division of surgical pathology of The University of Southern California School of Medicine, The New York Hospital-Cornell University Medical Center, and Memorial Sloan-Kettering Cancer Center. Cases conforming to the criteria for Bartholin's gland carcinoma suggested by Chamlian and Taylor[14] were selected. Briefly, on clinical examination the tumors were located in the region of Bartholin's gland, they arose deep to the vulvar skin, and were usually present as a distinct nodule. Histologically, the overlying vulvar skin was uninvolved and no other primary malignancies were present. In most cases, remaining Bartholin's gland ducts or acini could be identified in the vicinity of the neoplasm. Ten cases conforming to these criteria were identified between the years of 1966 and 1990.

Of the 10 cases of Bartholin's gland carcinoma identified, seven (70%) were squamous cell carcinomas, two (20%) were adenoid cystic carcinoma, and one (10%) was an adenocarcinoma. The mean age of patients at the time of diagnosis was 55 years (range, 39 to 84). In addition, blocks from four non-neoplastic Bartholin's glands excised during the course of surgery for non-neoplastic lesions were selected from our archives. These non-neoplastic glands were used as negtive HPV controls as well as for establishing the normal antigenic phenotype of Bartholin's gland. Well-studied cervical biopsies containing dysplastic squamous epithelium and HPV types 6, 11, 16, 18, and 33 were used in all *in situ* and PCR reactions as positive controls.

Immunohistochemistry

Formalin-fixed (10% buffered formalin), paraffin-embedded tissues were used. The avidin-biotin peroxidase conjugate method was used with minor modifications.[15] Briefly, tissue sections were cut onto slides coated with 0.005% poly-L-lysine (Sigma Chemical Co., St. Louis, MO) and incubated at 60 C for 2 hours. After rehydration, the endogenous peroxidase activity was quenched by incubation in 1% hydrogen peroxide (diluted in distilled water) for 10 minutes. The slides were washed in phosphate buffered saline (PBS) and exposed to protease digestion and/or detergent[16] as indicated in Table 1. After washing in PBS, sections were incubated in 5% horse serum/PBS for 1 hour at room temperature. This was decanted, replaced with appropriately diluted primary antibody (Table 1), and incubated in sealed moisture chambers for 12 hours at 4 C. The sections were then washed in PBS and incubated with biotinylated horse anti-mouse immunoglobulin (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. After washing in PBS, the sections were incubated with avidin-biotin complex (Dako, Santa Barbara, CA) at a 1:100 dilution in PBS for 1 hour at room temperature. After a final rinse in PBS, the sections were incubated in 0.06% 3,3′-diaminobenzidine (Sigma) in PBS with 0.003% hydrogen peroxide for 10 to 15 minutes. After counterstaining in modified Harris' hematoxylin (Fisher Scientific, Orangeburg, NY), the sections were dehydrated and coverslipped with Permount. The primary antibodies along with their working dilutions and commercial sources are listed in Table 1. Dilutions were in 2% bovine serum albumin/PBS. Also listed in Table 1 are the enzymatic/detergent pretreatments used.

In Situ *Hybridization*

Four to six micron sections from formalin-fixed, paraffin-embedded tissue were cut onto adhesive pretreated slides (ENZO Diagnostics, Syosset, NY), deparaffinized in xylenes, and rehydrated through serial alcohols to distilled water. Slides were digested with proteinase K (100 μg/ml) for 10 minutes and 0.5 mol/L hydrochloric acid for 10 minutes at 37 C, dehydrated through serial alcohols, and air

Table 1. *Primary Antibodies Used in this Study*

Antibody	Pretreatment/Digestion	Source*	Antibody Dilution
Cam 5.2	0.05% pepsin, 30 min	BD	1:100
B72.3	0.05% pepsin, 30 min	Biog	1:1000
CEA	0.05% saponin, 30 min	BM	1:750
EMA	0.05% pepsin, 30 min	Dako	1:500
MCA	0.025 trypsin, 7 min	ENZO	1:4000
Lewis X	0.05% saponin, 30 min	Sig	1:8
ER	Ficin	Ab	1:5
PR	none	Ab	1:10

* Antibody sources: BD: Becton Dickinson, San Jose, CA; Biog: Biogenex, San Ramon, CA; BM: Boeringer-Mannheim, Indianapolis, IN; Dako, Carpinteria, CA; ENZO, New York, NY; Sig: Signet Labs, Dedham, MA; Ab: Abbott, North Chicago, IL.

dried. Hybridization was performed utilizing the Patho Gene DNA Probe for Human Papillomavirus (ENZO Diagnostics) for the detection of HPV types 6/11, 16/18, and 31/33/51 as per manufacturer's recommendation. In the case of positive reactivity, additional sections were hybridized substituting the hybridization mixtures with DNA probes specific to individual HPV types (kindly provided by ENZO Diagnostics).

PCR

Ten micron sections from formalin-fixed, paraffin-embedded tissues were obtained for each case. The PCR was performed as described by Shibata et al[17] with modifications. Briefly, each section was deparaffinized through two 0.5-ml washes of xylene and two washes of 95% ethanol and speed vacuum dried. To this, 200 µl of distilled water was added and the mixture boiled for 10 minutes. The supernatant was collected and 20 µl used for each amplification reaction. The amplification reaction used was as recommended in the Perkin-Elmer Cetus Kit package insert with the following modifications: the concentration of magnesium was 2.5 mmol/L and the final volume of the reaction mixture was reduced to 50 µl. The recommended 2.5 units of Taq polymerase enzyme were used in each reaction. The final reaction mixture was overlaid with two drops of mineral oil and placed in a Perkin Elmer Cetus temperature cycler. The primers used for amplification were purchased from Oligos Inc., Guilford, CT, from the sequences designed by Class et al[18] and Shibata et al[17] for specific amplifications of HPV types 6, 11, 16, 18, and 33. Thirty-five cycles of the reaction were performed, each cycle consisting of 1 minute at 92 C (denaturation), 2 minutes at 42 C (annealing), and 3 minutes at 72 C (DNA synthesis). Each sample was also processed with primers designed to amplify a 180-bp segment of the β-globin gene under identical conditions to determine adequacy of the tissues for amplification. All samples were processed at least twice from initial extraction from paraffin to final hybridization, with consistent results. In addition, duplicate processing of all samples with two different sets of amplifying primers for HPVs types 16 and 18 was performed with consistent results.

PCR Product Analysis

Ten microliters of the postamplification products were electrophoresed on an 8% polyacrylamide gel. Positivity was determined by identification of a band of the expected length by ultraviolet flourescence of the ethidium bromide-stained gel. Positivity was then confirmed by Southern blot hybridization with specific HPV probes after 3% Nu-Sieve, 1% agarose gel electrophoresis of an addtional 10 µl of product, and transfer onto nylon filters (Bio-Rad, Richmond, CA). Filters were prehybridized in a mixture of 5X SSC, 20 mmol/L NaH_2PO_4, pH7, 7% SDS, 10X Denhart's, and 100 µg/ml of salmon sperm DNA at 50 C for 1 hour. This mixture was then replaced with 5×10^8 cpm of ^{32}P-labeled oligonucleotide probe in an identical mixture. Hybridization was allowed to proceed overnight at 50 C. Filters were then washed twice for 30 minutes at 50 C in 3X SSC, 10X Denhart's, 5% SDS, and 25 mmol/L NaH_2PO_4, pH 7.5, and once for 30 minutes in 1X SSC and 1% SDS, also at 50 C. Autoradiographs were exposed for 1 hour.

Results

Molecular Analysis

All specimens were amenable for DNA amplication by PCR as demonstrated by positive amplification of the 180-bp fragment of the β-globin gene (data not shown). HPV type 16 was amplified by PCR from six of the seven (86%) squamous cell carcinomas of Bartholin's gland (Figure 1). In one of these positive cases, HPV type 16 was also demonstrated by *in situ* hybridization (Figure 2). No other HPV types were identified in any of these cases by PCR or *in situ* hybridization. The remaining squamous cell carcinoma, the adenoid cystic carcinomas, the adenocarcinoma, and all four non-neoplastic Bartholin's glands were negative for all HPV DNA types tested by both PCR and *in situ* hybridization.

Immunohistochemical Results

The immunohistochemical reactivity of Bartholin's gland tumors and non-neoplastic glands is summarized in Table 2. Only four of the seven squamous cell carcinomas had sufficient remaining tumor tissue in the blocks to perform complete immunohistochemical studies. All other tumors were available for complete studies.

Cytokeratins

An antibody that recognizes low molecular weight acidic cytokeratins was used (Cam 5.2).[19,20] Cam 5.2 expression was not observed in the normal squamous epithelium overlying Bartholin's gland (Figure 3A), except focally in the basal cell layer. However, the antibody reacted with the transitional zone epithelium (Figure 3B) as well as ducts, acini, and myoepithelial cells of Bartholin's gland (Figure 3C). All

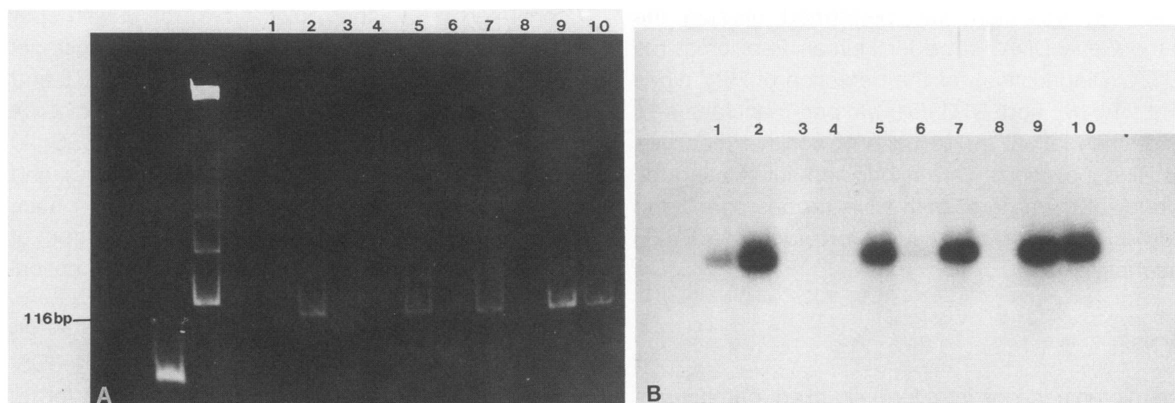

Figure 1. **A**: *Polyacrylamide gel electophoresis of PCR products showing positive amplification as seen by bands of 116 bp in lanes 2, 5, 7, 9, and 10. (Molecular weight marker: 123-bp ladder).* **B**: *Southern blot hybridization with specific HPV type 16 probe confirming positivity of electrophoretic results and revealing an additional positive case in lane 1. (autoradiography of ^{32}P-labeled DNA probe).*

squamous carcinomas, adenocarcinomas and adenoid cystic carcinomas tested showed reactivity with antibody CAM 5.2 (Figure 3, D-F).

B 72.3

The antibody B 72.3 reacts primarily with simple glandular and squamous epithelium.[21] Positive reactivity was seen in normal squamous epithelium, transitional zone, ducts, and acini of Bartholin's gland, whereas myoepithelial cells of the ducts and acini were negative. All squamous carcinomas and adenocarcinoma tested showed detectable levels of expression of B72.3, whereas adenoid cystic carcinomas were negative. Reactivity in positive tissues was generally focal.

CEA

CEA is expressed by squamous (Figure 4A) and transitional zone epithelium (Figure 4B), but not by ducts, acini, or myoepithelial cells of the normal Bartholin's gland (Figure 4C). The reactivity in transitional epithelium was usually focal (Figure 4B). Both squamous and adenocarcinomas showed reactivity with antibody to CEA (Figure 4, D and E), whereas adenoid cystic carcinomas were negative (Figure 4F) (focally, secretions were reactive with CEA).

Lewis X

Expression of Lewis X (a blood group related antigen expressed by epithelial cells)[22] was observed in transitional zone epithelium, ducts, and acini, and focally in squamous epithelium, but not in myoepithelial cells of the normal Bartholin's gland. Both squamous carcinomas and adenocarcinomas showed expression of Lewis X, whereas adenoid cystic carcinomas were negative.

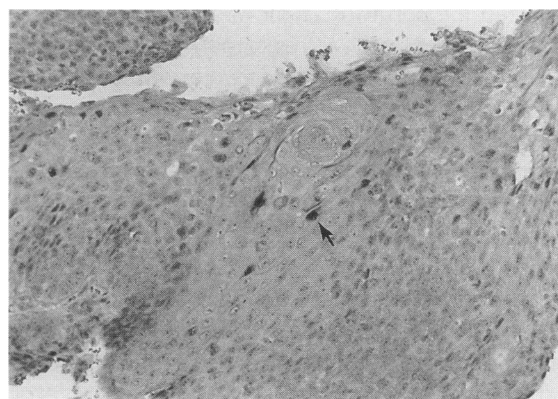

Figure 2. In situ *hybridization of a well-differentiated squamous cell carcinoma of Bartholin's gland showing positive reactivity (arrow) with a biotinyllated HPV type 16 probe.*

MCA (Muscle Common Antigen)

MCA (an antigen expressed by cells of myogenic origin[23]) is not expressed by squamous, transitional zone, duct, or acini epithelium of Bartholin's gland. It is expressed by myoepithelium of acinar cells and terminal ducts. Squamous, adeno, and adenoid cystic carcinomas do not show reactivity with antibody to MCA.

ER and PR (Estrogen and Progesterone Receptor)

Monoclonal antibodies specific for ER and PR were used to test for hormone receptor expression by the tumors.[24] Whereas the adenocarcinoma showed heterogeneous expression of both ER and PR, the squamous carcinomas and adenoid cystic carcinomas showed no detectable expression.

Discussion

We have demonstrated the presence of HPV type 16 DNA in squamous cell carcinomas of Bartholin's

Table 2. *Bartholin Gland Immunohistochemical Panel*

	Cam 5.2	B72.3	CEA	EMA	Lewis X	MCA	ER	PR
Squamous cell carcinoma (4)	4/4	4*/4	4*/4	4/4	3/4	0/4	0/4	0/4
Adenocarcinoma (1)	1/1	1/1	1/1	1/1	1/1	0/1	1/1	1/1
Adenoidcystic carcinoma (2)	2/2	0/2	0/2	2*/2	0/2	0/2	0/2	0/2
Non-neoplastic gland (4)								
Squamous epithelium	−	+	+	+	+*	−	NT†	NT
Transition zone	+	+	+	+	+*	−	NT	NT
Ducts and acini	+	+	−	+*	+	−	NT	NT
Myoepithelial cells	+	−	−	−	−	+	NT	NT

* Focal reactivity.
† NT, not tested.

gland. We have also shown that squamous cell carcinomas and adenocarcinomas of Bartholin's gland are antigenically similar to one another. These tumors are distinct from the normal squamous epithelium of the vulva and the ducts and acini of Bartholin's gland but are similar to the epithelium of the transition zone. Furthermore, we have shown that adenoid cystic carcinomas are antigenically distinct from both squamous cell carcinomas and adenocarcinomas and, therefore, may arise from a different cell type.

The association between squamous cell carcinomas of the genital tract and HPV has been well established.[25,26] Although numerous HPV types have been detected in squamous dysplasias of the uterine cervix, the majority of invasive squamous cell carcinomas contain HPV type 16 DNA.[26] Six of seven squamous carcinomas of Bartholin's gland in our series demonstrated HPV DNA by PCR and one of these showed HPV by *in situ* hybridization. The difference in the detection rate of HPV by the two techniques used can be explained by the respective sensitivities of the methods. In situ hybridization can only reliably detect 50 or more copies of viral genome in a cell, whereas PCR may be capable of detecting as few as a single copy.[17,27] This difference becomes significant in invasive carcinomas, where HPV is commonly integrated in the cellular genome and may be present in relatively few copy numbers per cell. The detection of HPV 16 in the neoplastic cells of one case of squamous cell carcinoma by *in situ* hybridization provides evidence that the viral genes are associated with the neoplastic process and that their detection is not caused by external contamination or to detection of HPV from adjacent nonmalignant HPV-infected cells.

Failure to detect HPV types 6, 11, 16, 18, or 33 DNA in one case of adenocarcinoma of Bartholin's gland is difficult to interpret. Recent studies have identified HPV DNA (particularly type 18) in adenocarcinomas of the uterine cervix, albeit at a lower frequency than in squamous cell carcinomas.[28] Our failure to identify HPV DNA in a single example of adenocarcinoma cannot be regarded as definitive. Inasmuch as both squamous cell and adenocarcinoma seem to be arising from the same cell type, it is attractive to postulate that, as in the cervix, both adenocarcinoma and squamous carcinoma of Bartholin's gland are associated with HPV infections. Additional work with larger numbers of adenocarcinomas and with probes to more types of HPV is needed to arrive at definitive conclusions.

The panel of antibodies used in this study was able to distinguish between the cells comprising the normal Bartholin's gland based on their antigenic phenotype. Squamous epithelial cells were found to express B72.3 and CEA but did not express detectable levels of Cam 5.2 (except focally in the basal layer). The cells at the transition zone expressed Cam 5.2, Lewis X, B72.3, and CEA, whereas the cells comprising the ducts and acini expressed Cam 5.2, Lewis X, and B72.3, but did not express detectable levels of CEA. As expected, myoepithelial cells were also antigenically distinct; they expressed Cam 5.2 and MCA, but did not express detectable levels of B72.3, CEA, or Lewis X.

Based on the antigenic phenotype of the cells comprising the normal Bartholin's gland, an origin of the carcinomas studied here could be postulated. We have found that both squamous and adenocarcinomas of Bartholin's gland have similar antigenic phenotypes, showing expression of Cam 5.2, B72.3, CEA, and Lewis X. These tumors, therefore, may arise from the cells of the transition zone (which shows the identical antigenic phenotype using the antibodies described here) and not from either the squamous epithelium (which is Cam 5.2 negative) or simple epithelium of the ducts and acini (which are CEA negative). This is similar with the origin of carcinomas arising in the uterine cervix.[19,20] The uterine cervix and Bartholin's gland have similar architectures, being comprised of squamous, transitional, and simple glandular epithelial elements. These different cellular elements within the cervix are antigenically distinct and show phenotypic similarities to the cells in the complementary zones of Bartholin's gland.[29-31] In particular, cervical squamous epithe-

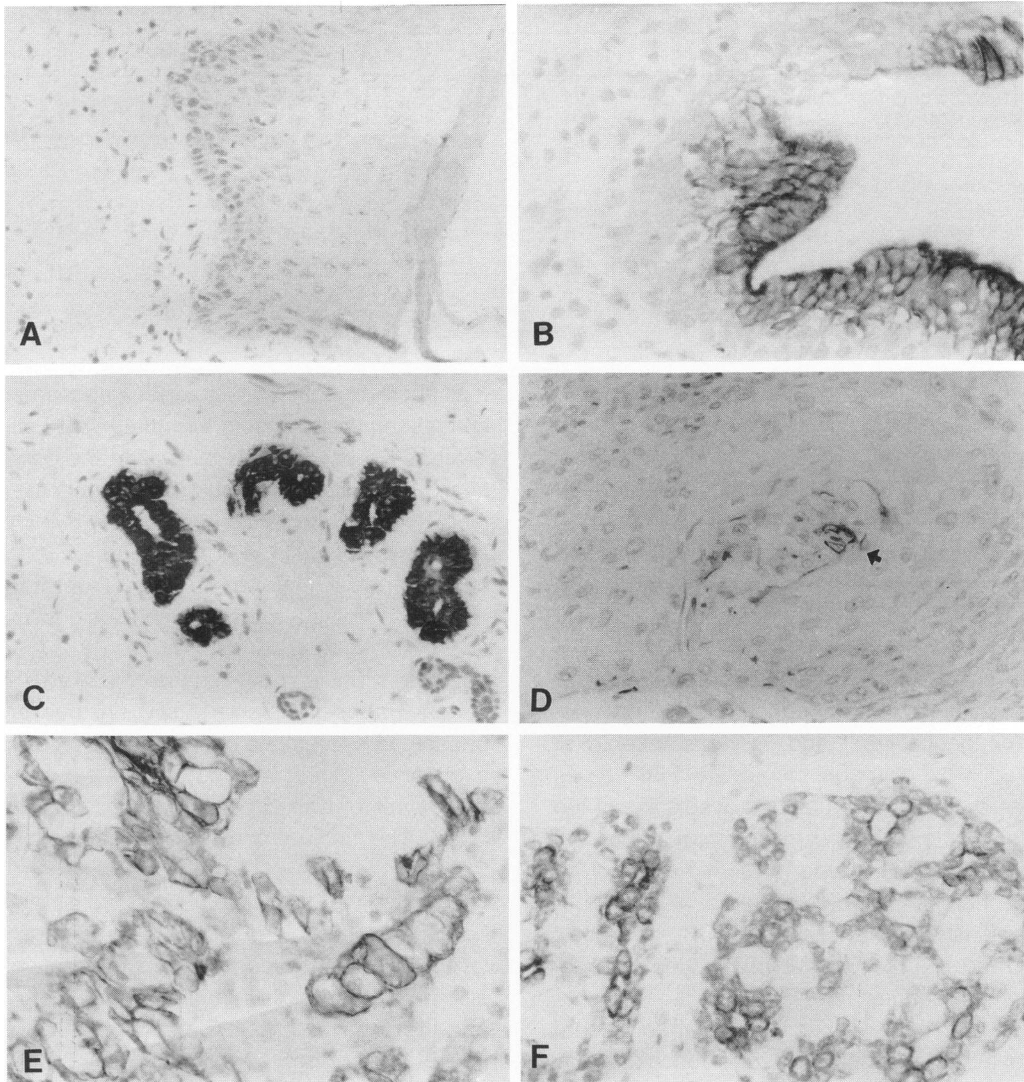

Figure 3. *Reactivity with antibody to cytokeratins (Cam 5.2).* **A**: *Squamous epithelium overlying Bartholin's gland. No reactivity seen (×100).* **B**: *Transition zone of Bartholin's gland, positive reactivity (×200).* **C**: *Duct/acinar structures of Bartholin's gland showing positive reactivity (×40).* **D**: *Squamous carcinoma of Bartholin's gland, exhibiting heterogeneous reactivity (arrow) (×100).* **E**: *Adenocarcinoma of Bartholin's gland, positive reactivity (×200).* **F**: *Adenoid cystic carcinoma of Bartholin's gland, positive reactivity (×100).*

lium does not express Cam 5.2, whereas the transition zone and cells of the endocervix do. Both squamous and adenocarcinomas of the cervix express Cam 5.2. Thus squamous and adenocarcinomas arising in Bartholin's gland and in the uterine cervix seem to have a similar origin in the transition zone. It is in the transition zone where cervical dysplasia and malignant transformation most commonly occurs.[32] It is of particular significance that squamous cell carcinomas of both Bartholin's gland and the uterine cervix seem to be highly associated with HPV infections as well.

The Bartholin's gland adenocarcinoma studied here showed detectable levels of hormone receptor expression (ER and PR). However, the four squamous carcinomas tested in this series did not show detectable hormone receptor expression. This is consistent with previous reports. It is interesting to speculate that carcinomas arising from the transition zone of Bartholin's gland (and perhaps of the cervix as well) show divergent differentiation to either squamous or adenocarcinomas based on their hormonal milieu or their hormone responsiveness.

Adenoid cystic carcinomas seem to be antigenically distinct from both squamous and adenocarcinomas of Bartholin's gland and thus may arise from a different cell of origin. Based on the panel of antibodies used in this study, the derivation of adenoid cystic carcinomas is difficult to ascertain. Adenoid cystic carcinomas do not seem to arise from either squamous epithelium (note differences in Cam 5.2, B 72.3, CEA, EMA, Lewis X, and MCA expression), transition zone epithelium (note differences in B 72.3, CEA, Lewis X, and MCA expression), or the simple

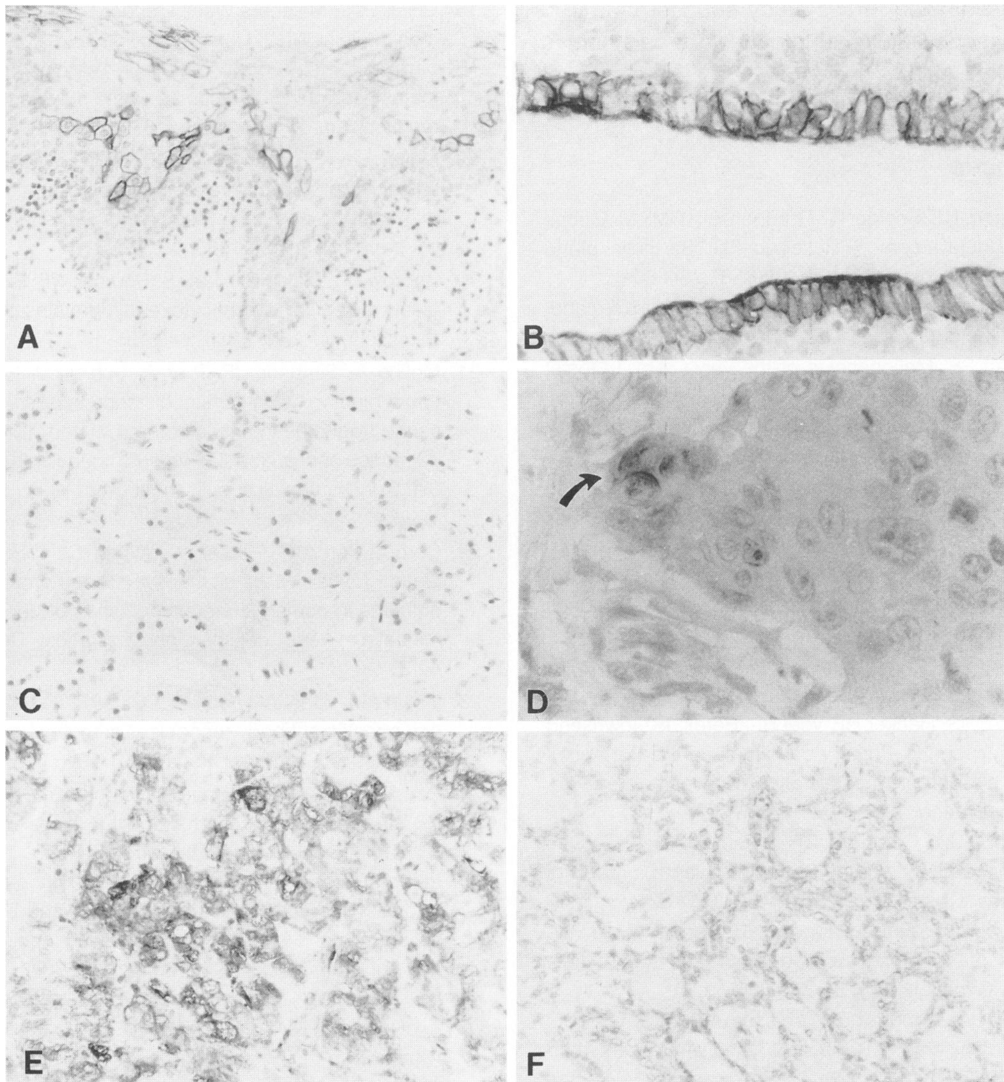

Figure 4. *Reactivity with antibody to carcinoembryonic antigen (CEA).* **A**: *Squamous epithelium overlying Bartholin's gland showing focal positive reactivity (× 100).* **B**: *Transition zone of Bartholin's gland, positive reactivity (× 100).* **C**: *Duct/acinar structures of Bartholin's gland. No reactivity seen (× 100).* **D**: *Squamous carcinoma of Bartholin's gland showing focal positive reactivity (arrow) (× 200).* **E**: *Adenocarcinoma of Bartholin's gland, positive reactivity (× 100).* **F**: *Adenoid cystic carcinoma of Bartholin's gland. No reactivity seen (× 100).*

epithelia of the ducts or acini (note differences in B72.3, EMA, Lewis X, and MCA expression). On the basis of the findings here, the origin of adenoid cystic carcinoma may be consistent with myoepithelial cells. Although this is consistent with previous reports,[33] the findings presented here cannot be considered conclusive because of the lack of complete concordance in the antigenic phenotype of myoepithelial cells and adenoid cystic carcinomas (in particular, the difference in MCA expression). It is particularly interesting to note that adenoid cystic carcinomas (at least the ones studied here) showed no evidence of infection with HPV or of ER/PR expression, in contrast to the tumors derived from the transition zone epithelium. This is further indication that they have a different origin.

We postulate that Bartholin's gland carcinomas are etiologically related to HPV infection, particularly with HPV type 16. Historical and epidemiological data suggest a sexually transmitted pattern, as evidenced by the rarety of occurrance in virgins and its association with carcinomas of the vagina and cervix. The low incidence of these tumors when compared with those occuring in the cervix may be explained by the more sequestered anatomic location of the Bartholin's gland when compared with the much more anatomically exposed and traumatized uterine cervix. The antigenic similarity between Bartholin's gland and the uterine cervix and between the tumors arising from them, and the identification of HPV type 16 in six of the seven squamous cell car-

cinomas examined in this study suggest that HPV may play a critical role in the genesis of carcinomas of Bartholin's gland.

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