Effect of Anal Epidermoid Cancer-related Viruses on the Dendritic (Langerhans') Cells of the Human Anal Mucosa

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ABSTRACT

Purpose: The incidence of anal cancer is high in patients with anal condyloma. HIV increases this risk. We analyzed anal mucosa from normal individuals and individuals with condyloma.

Experimental Design: Normal anal mucosa from 155 consecutively recruited patients (102 HIV-positive and 53 HIV-negative) was compared with that obtained from 30 HIV-negative patients after hemorrhoid surgery (controls). Langerhans' cells (LCs), T lymphocytes, and viruses [EBV, cytomegalovirus, herpes simplex virus 1, and human papillomavirus (HPV) types] in anal mucosa and HIV load and CD4 T-lymphocyte counts in the serum were characterized.

Results: None of the control individuals had anal squamous intraepithelial lesion or HPV versus 19 HIV-positive and 4 HIV-negative patients with anal condyloma ($P = 0.07$). The number of LCs/mm in anal tissue was significantly higher in HIV-negative patients with condylomata (median, 30; range, 2–130) than in HIV-positive patients (median, 15; range, 0–100) or in controls (median, 17; range, 4–35). In HIV-negative individuals, the occurrence of condylomata was linked with a higher number of LCs. Significant differences were observed between HIV-positive and HIV-negative patients with anal condylomata: number of LCs/mm anal tissue, oncogenic HPV (26% versus 8%), other current infections (35.6% versus 5%), being male (93% versus 74%). Multivariate regression analysis found HIV as the only risk factor for a decrease in the number of LCs (odds ratio, 6; 95% confidence interval, 2.28–16.1; $P < 0.001$) and the serum HIV load (odds ratio, 4.9; 95% confidence interval, 1.1–21.4 log/ml; $P < 0.03$) but not the serum CD4 T-lymphocyte rate as a predictive risk factor for having <17 LCs/mm tissue.

Conclusion: HPV increases the number of LCs in anal mucosa in HIV-negative individuals. HIV alters anal dendritic cells, likely leading to an increase in anal cancer risk.

INTRODUCTION

HPV is widespread in the homosexual population and causes anal condyloma. This lesion is considered to be a major epidemiological marker of individual risk for anal cancer (1–6). Indeed, squamous cell carcinoma is a rare neoplasm of the anal mucosa in the general population, and its incidence in homosexual, HIV-infected, and immunocompromised individuals is increasing (7–9). Anal condylomata increases the relative risk of anal carcinomas by 11.7 in women and by 8 in men (3).

HIV is another widespread infection in the homosexual population. It increases the relative risk of anal carcinoma by 1.7 in women and 3.1 in men (3). Furthermore, the frequency of relapses is significantly higher in HIV-positive patients than in HIV-negative individuals (10, 11).

The mechanism by which these two viruses induce anal carcinoma is unknown. HPV seems to induce high-grade dysplasia (HGSIL), probably by integrating into host DNA and/or inhibiting the p53 protein (12). HIV is thought to cause anal carcinoma by increasing the activity of HPV, particularly oncogenic types, by inducing acquired immunodeficiency syndrome (11). It is not clear whether and how the tissue immunity of the anal mucosa is altered in individuals infected with HIV and HPV. We addressed these issues by conducting a comparative study in consecutively recruited patients with anal condyloma. The anal lesions were resected in all cases and histologically analyzed. We also compared the number of immune cells, especially dendritic cells (also called LCs) in samples of normal anal mucosa from these patients with the number of immune cells in anal mucosa taken from healthy individuals undergoing surgery for hemorrhoids. We used statistical analyses to look at the effect of HIV status, HPV status, HPV oncogenic type, and systemic and anal mucosal immunity in the different groups.

PATIENTS AND METHODS

Patients and Study Design

All patients referred to the Departments of Coloproctology, Dermatology, or Sexually Transmitted Diseases of Bichat Hos-

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3 The abbreviations used are: HPV, human papillomavirus; HGSIL, high-grade squamous intraepithelial lesion; LC, Langerhans' cells; BMI, body mass index; CMV, cytomegalovirus; ASIL, anal squamous intraepithelial lesion; HSV, human simplex virus; ISH, in situ hybridization; OR, odds ratio; CI, confidence interval.
hospital with anal canal condyloma between January 1994 and December 1999 were recruited for a cohort study. Of the 155 patients recruited, 102 were HIV positive and 53 were HIV negative. Patients with invasive anal cancer were excluded. All enrolled patients were asked standardized questions about their sexual behavior, drug abuse, age, gender, BMI, sexually transmitted diseases, use and number of anti-HIV therapy. At enrollment, few (n = 9) patients had not yet received anti-HIV therapy, 15 were still receiving zidovudine monotherapy, and all others were receiving a combination of two or three drugs, zidovudine, stavudine, and lamivudine associated with indinavir, a protease inhibitor (n = 34). All of the patients underwent proctological examination to treat flat and acuminata condylomata in the anal canal and in the margin (anal area seen before anoscopy examination). Flat condyloma was defined as an acetowhite area or a slightly elevated tumor-like mucosa. They were white to pink in color. Condyloma acuminata were defined as exophytic lesions that could be easily distinguished from the normal mucosa. These lesions were classified as described previously (11). Treatment involved the excision of all lesions after the acetowhite test and careful reexamination under local or general anesthetic to check that the condyloma had been completely removed. Finally, two biopsies were taken from normal areas of the anal canal for immunopathological analyses.

Patients with Anal Condyloma

Two anal canal biopsies were taken from normal anal mucosa. The tissue obtained was used for histological and histochemical examinations. Each biopsy sample was divided into two; one half was fixed in formalin, and the other was frozen in liquid nitrogen. These samples were used for detection of viruses CMV, EBV, and HSV. Additional specimens or smears were obtained for the 20 HIV-positive patients who presented additional lesions (ulcer, fissure, or anoperineal abscesses). These lesions were classified as described previously (11). Treatment involved the excision of all lesions after the acetowhite test and careful reexamination under local or general anesthetic to check that the condyloma had been completely removed. Finally, two biopsies were taken from normal areas of the anal canal for immunopathological analyses.

Controls

Fresh anal mucosa samples were collected from normal areas in 30 patients suffering from hemorrhoids (n = 30; 22 males, 8 females) undergoing surgical treatment.

Histological Analysis

Condyloma, ASIL, and carcinoma were diagnosed using 4-μm-thick, paraffin-embedded sections of formalin-fixed tissue stained with H&E-saffron. The periodic acid-Schiff, Ziehl, and Grocott methods were also used to detect microbiological agents. Condyloma was diagnosed according to standard histological criteria (13, 14): the presence of papillomatosis associated with hyperacanthosis and the presence of koilocytes in the large cells of the epithelium, with double, dense, pyknotic, or folded nuclei, and perinuclear halos. Superficial para- or orthokeratosis was associated in some cases. The appearance of “glycogenic acanthosis” was suggestive of flat condyloma. Low grade squamous intraepithelial lesions (also called “grade I-II anal intraepithelial neoplasia” or “low-grade ASIL”) and high-grade squamous intraepithelial lesions “also called grade III anal intraepithelial neoplasia” or “high-grade dysplasia (HGSIL)” were defined as described previously (11). A histological examination did not reveal significant mucosal injury or major vascular dystrophy in the controls.

Immunohistochemistry for Normal Anal Mucosa

Antigen-presenting Cells and T Lymphocytes. The adjacent sections of anal mucosa were first stained with H&E to detect any mucosal abnormalities. LCs and CD3, CD4, and CD8 lymphocytes were then counted in frozen sections using the three-step peroxidase technique. Monoclonal antibodies directed against CD8 (labeling T cytotoxic/suppressor cells), CD3 (T lymphocytes), CD4 (T helper/inducer cells), CD22 (B lymphocytes; all from Becton Dickinson, Mountain View, CA) and CD1a (LCs; Immunotech, Marseille, France) epitopes were used. Between 5 and 12 serial sections (entire thickness of the epithelium without skin appendages, cut perpendicularly and taken from two different sites of mucosa) were analyzed for each factor and each patient. Each experiment included negative controls in which the primary antiserum was replaced with PBS and positive controls in which representative sections of skin were stained in a similar manner.

Morphometric Analysis

LCs. The number of CD1a-labeled cells per/mm was determined by use of an ocular grid (15). Immunostained cells were counted in 10 consecutive fields at ×400 (i.e., for a 2.8-mm-long anal epithelium sample in each case) on tissue sections from two different sites. Results are expressed as the number of CD1a/mm tissue.

T Lymphocytes. Cells immunostained with antibodies directed against CD3, CD4, CD8, and CD22 were also counted. Because LCs were also labeled with the anti-CD4 antibody, only round CD4-labeled cells that had an intensely stained cytoplasm were considered to be lymphocytes.

Immunohistochemical Detection of CMV at Enrollment

A mouse monoclonal antibody directed against CMV clone E13 was used (Biosys, Compiegne, France). The primary antibody was diluted 1:50 and incubated with the sections for 1 h at room temperature. The sections were then incubated with the secondary biotinylated horse antirat IgG (Vector Labs, Burlingame, CA) and the avidin-biotin-peroxidase complex (Vectorstain ABC kit; Vector Labs). The specificity of the immunostain was investigated by omitting the primary antibody.

Detection of HPV, EBV, and HSV

Characterization and Labeling of Primers and Molecular Probes. For HPV, the 20-mer MY11 and MY09 (Perkin-Elmer, Norwalk, CT) were used as primers for in situ PCR.
These primers correspond to a region of the major L1 protein of the viral capsid that is common to 40 HPV types. For ISH, biotinylated and FITC-labeled genomic DNA probes were purchased from Argène (Varhiles, France) and Dakopatt (Glostrup, Denmark). The probes were labeled by nick translation. Two kinds of HPV DNA probe were used. One was a mixture of various genomic HPV DNAs and was used to screen for the presence of HPV. The other probes were specific for the various types of HPV: 6, 11, 16, 18, 31, and 33. These probes corresponded to the complete DNA genome of each HPV type, inserted into pBR322. For EBV, a biotinylated oligonucleotide probe (Argène) complementary to the two nuclear EBER RNAs, was used. For HSV, a biotinylated genomic DNA probe (Enzo Diagnostic, Farmingdale, NY) corresponding to a mixture of two clones, one of HSV1 and one of HSV2 DNA, was used.

**ISH and PCR-ISH for the Detection of Viruses.** For HPV screening and typing, ISH and PCR-ISH were performed according to our protocol published previously (10). PCR-ISH was used only for samples that appeared HPV negative by ISH alone. For EBV and HSV, 20 ng of probe diluted in the same hybridization mixture as used for HPV detection was applied to each slide. Hybridization was performed in a moist chamber for 18 h at 37°C. Sections were washed three times in 0.1 × SSC at 37°C, then at room temperature, according to the probe manufacturer’s recommendations. For *in situ* detection, slides with biotinylated probes were incubated with streptavidin and then with biotinylated alkaline phosphatase (Argène). Slides with FITC-labeled probes were incubated with mouse anti-FITC antibody and then with rabbit antimus mus immunoglobulins and finally with a monoclonal mouse antirabbit antibody linked to the alkaline phosphatase anti-alkaline phosphatase complex (Dakopatt). For both kinds of probe, alkaline phosphatase activity was detected using the nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate chromogenic substrate. The hybridization specificity was determined by omitting the probes, and the specificity of PCR was determined by omitting the primers.

**Statistical Analysis**

The characteristics of the patients were recorded, using counts and percentages for categorical variables, and median and range or mean ± SD, as appropriate, for continuous variables. The following subgroups were identified: individuals with and without condyloma, with and without LGD or HGSIL, HIV-positive, and HIV-negative. Categorical variables were compared between groups using the χ² test or the Fisher’s exact test as appropriate. Continuous data were compared using Student’s *t* test or the Mann-Whitney *U* test, as appropriate. Differences between groups were considered to be significant if *P* < 0.05. All significant characteristics were considered to be univariate risk factors. To identify independent risk factors for anal immune cell alteration, we included all univariate risk factors in a multiple logistic regression model. The final model was constructed using a stepwise selection procedure. Accordingly, LC deficiency was defined as <17 cells/mm anal mucosa, which was the median value in normal individuals. Risk factors were constructed using a stepwise selection procedure. Accordingly, LC deficiency was defined as <17 cells/mm anal mucosa, which was the median value in normal individuals. Risk factors were identified for LC variations. The study was approved by the local ethical committee: Comité Consultatif de la Protection des Personnes se prêtant à une recherche biologique.

**RESULTS**

Of the 185 individuals included in the current analysis, 102 were HIV positive and 83 were HIV negative (53 with anal condyloma and 30 without who were considered as controls). The main characteristics of the individuals are shown in Table 1. Of the HIV-positive patients, 80% were Caucasian, 93% were male, 86% declared having had anal intercourse, and 33% had at least one other current anal infection (24% HSV, 12% CMV, and range or mean ± SD, as appropriate, for continuous vari-

### Table 1 Characteristics of individuals

<table>
<thead>
<tr>
<th>Control</th>
<th>With condyloma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>30</td>
<td>53</td>
</tr>
<tr>
<td>Age, yr</td>
<td>(18.3–37)</td>
<td>(18–34)</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>21/9</td>
<td>41/12</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>20.5</td>
<td>22</td>
</tr>
<tr>
<td>Median (range)</td>
<td>(18.3–27)</td>
<td>(18–34.5)</td>
</tr>
<tr>
<td>Anal intercourse</td>
<td>0</td>
<td>41a</td>
</tr>
<tr>
<td>Drug abuser</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Other infections</td>
<td>0</td>
<td>4a</td>
</tr>
<tr>
<td>Dysplasia within condylomas</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>HGSIL</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>LGD</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>HPV (6, 11)</td>
<td>38</td>
<td>81</td>
</tr>
<tr>
<td>HPVonc (16, 18, 31, 33)</td>
<td>7a</td>
<td>26a,b</td>
</tr>
<tr>
<td>LC/mm, median (range)</td>
<td>17 (4–35)</td>
<td>30 (2–130)</td>
</tr>
<tr>
<td>T-lymphocytes/mm, median (range)</td>
<td>17 (1–29)</td>
<td>10 (0–74)</td>
</tr>
<tr>
<td>CD3</td>
<td>7.1 (4–11)</td>
<td>5 (0–75)</td>
</tr>
<tr>
<td>CD4</td>
<td>14 (8–22)</td>
<td>7 (0–36)</td>
</tr>
</tbody>
</table>

*a* Significant difference compared with the control, *P* < 0.01.

*b* Significant difference compared with the HIV-negative patients presenting with anal condyloma, *P* < 0.01.
and 8% EBV). The mean time since the first positive HIV test was 31 (±40) months. The proctological examination at baseline showed no condyloma in the margin or anal canal in the 30 HIV-negative individuals considered as controls. However, in all other individuals, condylomata were observed in the anal canal with possible extension to the margin. There was no significant difference in the type, density, and distribution of condylomata according to HIV status. Condylomata were found particularly common in patients who declared having had anal intercourse. The rate of additional anal infection to HPV was significantly higher in the patients with condylomata than in the controls (24.5% versus 0%; \( P = 0.01 \)), and HIV had a significant effect (Table 1).

As expected, there was more ASIL within condylomata in HIV-positive patients (without significant link with number and combinations of anti-HIV drugs) than in HIV-negative patients (18.6% versus 7.5%; \( P = 0.043 \); Table 1). Oncogenic HPV, including types 16, 18, 31, and 33, was more common in HIV-positive patients than in HIV-negative patients (Table 1).

**Immune Cells in Anal Tissue**

**LCs.** CD1a-positive cells were found in the stratified epithelium in all samples. In most of the specimens, CD1a was arranged in an interconnected network at the suprabasal layer and exhibited the characteristics of dendritic cells (Fig. 1). In several cases, we observed LCs in the basal layer and in the lamina propria. In a small number of cases, the LCs were surrounded by T lymphocytes (CD4 and CD8 stained cells) displaying a rosette-like pattern.

The density of LCs could vary from one site to another, and results expressed are the mean of the density of different anal mucosa sites. Morphometric analysis showed 17 LCs/mm of anal mucosa in the control group (Fig. 1). The density of LCs was significantly higher in HIV-negative individuals with anal condyloma (Fig. 2) and significantly lower in HIV-positive patients with anal condyloma.

When all individuals (\( n = 185 \)) were considered, the density of LCs was not found to be linked to age (\( r = 0.27; P = 0.11 \)), gender, or BMI. HIV-positive patients and patients with multiple additive anal infections were significantly more likely to have \(<17 \text{ LCs/mm of mucosa (Table 2). Multivariate analysis, including all parameters with } P = 0.1 \text{ after univariate analysis, showed that only an HIV-positive test (OR, 4.5; 95% CI, 1.88–10.6; } P = 0.007 \text{) was a significantly higher individual risk factor for having a below average number of anal LCs.}

When all of the HIV-negative individuals (\( n = 83 \)) were considered, simple regression analysis did not find a significant difference between those with and without anal condyloma in terms of ethnic origin, age, gender, or BMI. In a multivariate analysis, including LCs, age, gender, anal intercourse, and additive current anal infection, only anal condyloma (\( P = 0.0001 \)) was found to be an independent factor (OR, 10; 95% CI, 3–35) for having an increased number of LCs in the anal mucosa.

When all of the individuals with anal condyloma were considered (Table 3), HIV-positive individuals were shown to have 6-fold higher risk of having a below average number of LCs in the anal mucosa. We tested several parameters in a univariate analysis and found that the HIV load in the serum and multiple anal infections were significant parameters to predict a decreased density of anal LCs in HIV-positive patients (Table 4). However, only HIV load was an independent risk factor for a decreased number of LCs. In HIV-positive individuals, the density of LCs in the anal mucosa, but not the serum level of T-lymphocyte CD4, was found to be inversely correlated with serum HIV load (Fig. 3).

**T-Lymphocytes in the Anal Mucosa.** T-lymphocyte CD3, T-helper/inducer cells (CD4), and T-cytotoxic/suppressor cells (CD8) were observed in the anal epithelium in all analyzed samples, mainly located at the basal layer. T-lymphocytes were widespread in the lamina propria, where they formed clusters or a rosette-like pattern. The CD4 lymphocytes in the lamina propria were predominant in most of the samples, with no

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**Fig. 1** LCs in the anal mucosa in a HIV-negative individual without condyloma. Immunohistochemistry was performed on anal mucosa from the anal canal using a CD1 polyclonal antibody in the control. \( \times 400 \).
significant difference between groups. Nevertheless, the mean number of intraepithelial T-lymphocytes was lower in HIV-positive patients than in the other groups (Table 1). However, taking all HIV-positive individuals together, a significant correlation could not be established between number of circulating and mucosal T-lymphocyte CD4 and CD8 ($r = 0.17$ and $r = 0.19$, respectively). There was no linear correlation between the number of LCs and the number of CD4 or CD8 cells in the anal mucosa of HIV-positive patients, although LCs (13.4 ± 1.7 versus 20.2 ± 2.2 cells/mm; $P = 0.01$) and CD4 lymphocytes (4.7 ± 0.6 versus 5 ± 0.5 cells/mm; $P = 0.01$) were significantly lower in HIV-positive patients than in those without HSIL.

**DISCUSSION**

This comparative cohort study showed that HPV-infected patients have more LCs in their anal mucosa than normal individuals. HIV infection was shown to suppress this effect.

We have previously characterized LCs and T lymphocytes CD3, CD4, and CD8 in the normal anal mucosa (15). LCs are a type of dendritic cells. Intraepithelial LCs act as antigen-presenting cells for intraepithelial lymphocytes (15–17) in human anal mucosa. The number of LCs in the anal mucosa may increase during inflammatory conditions or mucosal infections (18, 19), in particular in the HPV-infected tissue (20). LCs are involved in first-line management of antigens (16). In this study,
The incidence of anal canal carcinoma has increased over the last 20 years, particularly in men. The increasing prevalence of sexually transmitted diseases, in particular HPV, seems to account for this epidemiological phenomenon. HPV infection is strongly linked with the development of anogenital lesions i.e., condyloma (22). It is now well established that of the factors linked with sexually transmitted diseases (anal condyloma, anal intercourse, multiple anal infection, and HIV-positive status), anal condyloma is the main risk factor for anal carcinoma (1–3). Patients coinfected with HIV and HPV have a higher risk of developing ASIL, the step before carcinoma and cancer (10, 23–26). Although, oncogenic HPV types (27–31) have been reported in anal carcinoma, they could not be always identified as an independent risk factor for anal HGSIL and cancer (10). Thus, the production of oncogenic viral proteins alone is probably insufficient to lead to the development of invasive carcinoma. Furthermore, HIV-positive patients are more likely to have recurrent condyloma and HGSIL than HIV-negative patients, regardless of the HPV type (10). This suggests that the immune parameters have been altered i.e., T-lymphocyte CD4 in the serum may be an alternative mechanism. Indeed, among HIV-positive men, the prevalence of HPV increases as the CD4 count decreases, suggesting a strong relationship with HIV-associated immunosuppression (7). However, the reason for the neoplastic transformation of such lesions remains unclear. Because HPV is the main cause of anal carcinoma and immunodepression per se [HPV-infected patients (32) and drug addicts (33–35)] and increases the risk of anal carcinoma development, we might suggest two steps evolving anal carcinoma. The first step is characterized by HPV remaining in the anal mucosa, even after condylomata has been cured. This is likely attributable to T-lymphocyte diminution in the anal tissue because either CD3, CD4, or CD8 tissue lymphocytes appear to be decreased in HPV-infected patients versus control individuals (Table 1). The second step is characterized by dendritic cell alteration i.e., HIV infection) coming with condyloma relapses.

Our data plus the fact that HIV influences the expression of HPV genes (36) explain why HPV alone cannot explain the carcinogenesis. Indeed, HIV resulting in local immunosuppression might lead to an inappropriate immune surveillance of viral infection. This might explain the higher rate of dysplasia and cancer in the perianal area (37). We have shown previously (10) and confirm currently that HGSIL is associated with HIV load in the serum and not number or combination type of anti-HIV drugs. In addition, a diminution of LCs in HPV-infected tissue has been reported by others in HIV-positive women, in particular those developing cervix carcinoma (38–40). More specifically, a decrease in the number of LCs has been suggested to be linked to the degree of dysplasia (10, 41–43). Thus, HIV may increase HPV activity by reducing the number of immune cells in the tissue. This is consistent with the fact that HIV increases the turnover rate of HPV in the anal tissue (36) and by the fact that the level of immune cell modification in the tissue is dependent on the HIV load. In a recent study, Miyagi et al. (20) showed higher infiltration of LCs in all HPV-infected carcinoma than in non-HPV-infected carcinoma of the lung. That LCs increase in the lung tissue is further linked with a better prognosis (20).

In summary, we found a higher density of antigen-present-
ing cells in the anal mucosa of HIV-negative patients with anal condyloma and a lower density in HIV-positive patients. These findings are not related to gender, age, multiple anal infection, or sexual behavior. HIV inhibits the stimulation of LCs by HPV in a density-dependent manner. These findings may elucidate the causes of condyloma relapse and their transformation into cancer.

REFERENCES


18. Romito, A., Grizzuti, M. A., Tucci, M., and Silvestris, F. Malignant neoplasms and AIDS. Review of the literature and critical consider-
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