**Abstract**

**Purpose:** Increasing risk of squamous cervical intraepithelial neoplasia (CIN) exits in HIV-infected women. However, the relatively low incidence of invasive carcinoma in the untreated HIV-infected population suggests an imbalance between cell proliferation and apoptosis. We investigated apoptosis and caspases in cervical samples from this population comparatively to non-HIV-infected and control subjects.

**Experimental Design:** Apoptotic terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling method, immunohistochemistry for caspase-2, caspase-3, caspase-8, caspase-9, and other apoptosis markers were done on 12 normal cervical samples and 103 low- and high-grade cervical lesions, containing human papillomavirus(es) from 35 HIV-negative and 33 HIV-positive women before tritherapy advent.

**Results:** (a) The apoptotic index (AI) in epithelial cells did not vary between normal mucosa and condyloma acuminata infected or not with HIV. (b) AI augmented with the CIN severity in HIV-positive and HIV-negative women. (c) AI dramatically increased in oncogenic human papillomavirus-infected CIN of HIV-positive population compared with the CIN of similar grade in HIV-negative one. This was associated with a greater expression of caspase-8, active caspase-9, and active caspase-3 in those samples. Moreover, densities of Langerhans’ cells, involved in apoptotic bodies engulfment, were greatly reduced in CIN of HIV-positive women. In samples, these densities were highly inversely correlated with AI (r = −0.88, P < 0.002).

**Conclusions:** This study provides the first evidence for the strongly enhanced apoptosis levels and caspase expression in CIN of untreated HIV-infected women. We suggest that the reduction in Langerhans’ cell number could contribute at least partly to apoptotic cell accumulation.

Physiologically, cells are eliminated by programmed cell death or apoptosis. Regulation of this process is a complex machinery. This one involves among others effectors (i) several proto-oncogenes or tumor suppressor genes as p53; (ii) proteins of the Bcl2 family which are key regulators of apoptosis and either death antagonists such as Bcl2 members or death agonists such as Bax members; (iii) caspases (cyteinyl aspartate–specific proteinases) which are apoptosis executive enzymes and the central mediators of the proteolytic cascade leading to death and elimination of cells. A decisive event during apoptosis is their activation from their zymogenic proforms. Some of them are initiators such as caspase-2, caspase-8, and caspase-9 and are situated at the top of the cascade. Others such as caspase-3 are activated by another caspase and are downstream executioners. Apoptotic cell fragments can be eliminated by engulfment assured by macrophages or immature dendritic cells, among them Langerhans’ cells (1–3). Normal cellular homeostasis is maintained by the balance between proliferation and apoptosis. Genetic events leading to an increase in proliferation as well as failure of tumor cells to undergo apoptosis can result in uncontrolled accumulation of cells and therefore in cancer development.

Carcinogenesis in the cervical epithelium is characterized by the occurrence of successive events, from the disturbance of epithelial cell proliferation and/or differentiation via low- and high-grade cervical intraepithelial neoplasia (CIN), to invasive carcinoma. Human papillomavirus (HPV) has been implicated in the development of CIN (4). All genital condylomas and most intraepithelial neoplasia and invasive cervical cancers contain HPV DNAs (4). HPV types 16, 18, 31, and 33 are most commonly found in cervical neoplasia whereas types 6 and 11 are linked to condyloma acuminata or regressive dysplasia. Studies of cell proliferation and apoptosis in the cervical mucosa of women in normal and pathologic conditions have been done with the Ki-67 labeling and the end labeling of DNA strand breaks [in situ end labeling or terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) methods], respectively. They have shown that the apoptotic index (AI) significantly increases.

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Received 7/9/04; revised 7/12/04; accepted 11/1/05.

**Grant support:** Agence Nationale de Recherche pour le Sida grant 97005 (F. Walker) and Institut National de la Santé et de la Recherche Médicale.

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with the progression of cervical neoplasia through CIN to carcinoma and is not influenced by the presence of HPV in lesions (5–9). For some authors, cell proliferation evolves parallel to the AI (5, 8). Caspase-3 seems to be implicated in apoptosis in normal and pathologic cervix (9, 10).

HIV is an infection which per se increases the risk of carcinogenesis (11, 12). In that disease, apoptosis has been largely reported to occur in CD4 lymphocytes of the immune system (13–16). Recently, in HIV-associated pathologies, increased apoptosis has also been described in neurons (17–19), vascular endothelial cells (20), and cardiomyocytes (21). Patients with HIV infection have been shown to present a high prevalence of both cervical HPV infection and CIN. Nevertheless, during the follow-up of one cohort of HIV-positive women before the tritherapy advent, we noted a rather low incidence of invasive cervical cancers despite a high frequency of high-grade CIN (22). This was intriguing and led us to evoke a positive imbalance in favor of apoptosis over cell proliferation.

Therefore, in the present study, we investigated the apoptosis in the cervical lesions of tritherapy untreated HIV-infected women in comparison with that in the normal mucosa and cervical lesions from HIV-negative women. This was done using the TUNEL method, immunohistochemistry for caspase-2, caspase-3, caspase-8, caspase-9, and other apoptosis markers, and morphoquantitative techniques. Because Langerhans’ cells display morphologic changes and reduced numbers in squamous lesions from HIV-infected patients (23–25), we also estimated their density in the same cervical tissue samples and analyzed their relationship with AIs.

### Materials and Methods

#### Patients and tissues. Specimens of squamous cervical lesions infected with HPV(s) were obtained from 68 consecutive women during 1996. Eleven had condyloma acuminata containing non-oncogenic HPV types 6 and 11, and 57 had intraepithelial neoplasia containing oncogenic HPV. In situ hybridization and PCR-in situ hybridization were used for HPV screening and typing, with PCR-in situ hybridization done only for samples which seemed HPV negative for in situ hybridization alone (ref. 26, see for detail). Thirty-five of the 68 women were HIV negative. Thirty-three women were HIV-positive and were treated at the Bichat-Claude Bernard Hospital, Paris, before occurrence of tritherapy. HIV status was confirmed by Western immunoblotting. Forty-six percent of HIV-positive women before the tritherapy advent, we noted a rather low incidence of invasive cervical cancers despite a high frequency of high-grade CIN (22). This was intriguing and led us to evoke a positive imbalance in favor of apoptosis over cell proliferation.

All HIV-positive patients underwent colposcopy and systematic biopsies, whereas HIV-negative patients with condylomas were selected on the basis of Papanicolaou smear examination followed by colposcopy and biopsies. For all patients included in the study, microbiological tests for Chlamydia, Gonococcus, Mycoplasma, Mycobacterium and serologic tests for syphilis were negative. All tissue samples were routinely formalin-fixed, paraffin-embedded and cut into 4-μm-thick sections. Several sections were obtained from each block; the first were stained with H&E saffron for histologic diagnosis and subsequent sections were used for the detection of HPV and other viruses and for detection of apoptosis and factors possibly involved in apoptosis.

**In situ labeling of apoptotic cells by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling method.** Apoptotic cells were identified by a terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) method. After dewaxing, tissue sections treated with proteinase K (20 μg/mL) for 10 minutes at room temperature and washed in PBS, TUNEL reaction was used according to procedures provided with the ApopTag Plus Peroxidase. In situ Apoptosis Detection Kit (Serologicals Co., Biotech, Norcross, GA). Briefly, after adding equilibration buffer, the sections were exposed to TdT with digoxigenin-11-dUTP and dATP in a moist chamber for 60 minutes at 37°C. Antidigoxigenin-peroxidase was then applied to the slides. Peroxidase activity was revealed with diaminobenzidine substrate. Tissues were counterstained with hematoxylin. To confirm the specificity of the TUNEL method, a negative control was run, omitting TdT from the reaction mixture. As a positive control, sections of normal human lymph node tissues were used.

**Determination of the apoptotic index.** The AI in the normal mucosa, condyloma acuminata, and CIN lesions was estimated by two independent observers (F.W. and T.L.) examining the same cervical sample at the same time and using an ocular grid at ×400 magnification (×40 objective and ×10 ocular). For each tissue sample, representative areas of the reaction in the normal mucosa or lesions were chosen. Starting at one end of these areas, the cell count represented the number of positively labeled nuclei with apoptotic morphology (i.e., shrunken nuclei, including apoptotic bodies) and the total number of nuclei seen throughout a full-thickness epithelial column, the width of which was that of the grid. The field was then shifted and a new count done until an average of 1,000 cells (900-1,500 cells) was obtained. Cells in the vicinity of necrotic or inflammatory areas were not assessed. The AI was expressed as percentage of the total number of epithelial cells counted. In cases of unusual elevated AI (i.e., in samples of some HIV-positive patients), data were verified by performing a second count at several week intervals and for some cases, a new TUNEL reaction was done.

**Immunostaining for caspases, Fas and Fas ligand, Bcl-2, and p53.** Tissue sections were incubated with the following antibodies: mouse monoclonal antibodies (Novoceastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom) against Fas or Fas ligand diluted 1:25 and 1:20, respectively and against human caspase-2, caspase-3, or caspase-8 recognizing the proform of each enzyme diluted 1:10, 1:20, and 1:30, respectively, polyclonal affinity-purified rabbit IgG directed specifically against the active form of human caspase-3 (R&D Systems Europe Ltd., Abingdon, United Kingdom) diluted 1:100, or against the active form of human caspase-9 (BioSource, Camarillo, CA) diluted 1:20. Mouse monoclonal antibodies against human Bcl-2 oncprotein and human p53 protein (Dakopatt, Gloseur, Danemark) were also applied at a 1:40 and 1:50 dilution, respectively. Then, sections were incubated with the corresponding biotinylated secondary anti-mouse or anti-rabbit IgG diluted 1:200 and finally with the Elite complex diluted 1:20 to 1:100, depending on the primary antibody (kit Elite PK6100 Vector,Vector Laboratories, Burlingame, CA). Immunoreactivity was revealed by diaminobenzidine and nuclei were counterstained with hematoxylin. Immunostaining specificity was checked (a) by omission...
of the primary antibody, (b) by replacing the latter by control isotype immunoglobulin (i.e., mouse IgG1 or rabbit IgG; R&D systems) or by another irrelevant antibody with the same isotype (i.e., rabbit IgG anti-Helicobacter pylori; Dakopatt). In all these cases, no typical signal was seen in cervical tissues.

Immunostaining of Langerhans' cells. Langerhans’ cells were identified by immunostaining with a non diluted mouse monoclonal CD1a antibody (Immunotech, Marseille, France). Positive controls were representative sections of skin stained in the similar manner. The number of CD1a-labeled cells was determined in each lesion using an ocular grid by counting positive cells at least in four consecutive fields (depending on the length of the lesion) at ×400 magnification. Results were expressed as the number of CD1a per mm of mucosa.

Ki-67 antigen labeling. The Ki-67 antigen is a nuclear protein present in all stages of the cell cycle, except G0. Some tissue sections containing normal mucosa and CIN were immunohistochemically stained, after antigen enhancement by immersing the slides in a sodium citrate buffer and heating in a steamer for 40 minutes, using a mouse monoclonal antibody Ki-67 (clone MIB-1, Dakopatt) diluted 1:100. Proliferative index was roughly estimated as being 0% to 25%, 25% to 50%, and >50%.

Statistical analysis. Quantitative results were expressed as means ± 1 SE. Differences between two groups were evaluated with the Student’s t test or the Mann-Whitney U test whenever relevant. Correlations were estimated with the linear regression or the non parametric Spearman’s rank correlation. P < 0.05 (two-tailed tests) were considered statistically significant.

Results

HIV infection is associated with increased apoptotic indices in pathologic cervical mucosa. Mean AI in the different groups of patients are given in Table 1. In the normal mucosa of healthy women, the mean AI was <1. Positively labeled nuclei were seen essentially at the mucosal surface, more rarely in the depth of mucosa (Fig 1A). A total of 103 squamous cervical lesions containing HPVs were analyzed (i.e., 13 condylomas acuminata and 90 CIN). All three grades of CIN were observed in HIV-positive and in HIV-negative women. We considered CIN1 as low-grade squamous intraepithelial lesion and we grouped together CIN2 and CIN3 as high-grade ones. The number of CIN1 and CIN2/CIN3 lesions were similar in the groups of HIV-negative and HIV-positive patients as well as within a given group. In HIV-negative patients, mean AI in condylomas acuminata and in CIN1 was low and roughly of the same order as in the normal cervical epithelium of healthy women. In CIN2/CIN3, AI was significantly higher than in controls (P < 0.005; Fig. 1B; Table 1). In HIV-positive patients, AI was still low in condylomas acuminata but tended to increase as compared with corresponding values in HIV-negative patients. In CINs of these patients, AI was dramatically enhanced (P < 0.0001) by comparison with lesions of similar grade in HIV-negative patients. Apoptotic cells were often grouped in foci (Fig. 1C-D). When, in HIV-negative and HIV-positive groups, only the patients presenting the two types of low-grade (CIN1) and high-grade (CIN2/CIN3) lesions were considered, the same significant increase of AI was found in CINs of HIV-positive patients compared with corresponding values in HIV-negative ones (Table 1). Figure 2 shows all individual values of AI observed in each type of cervical lesions. The highest AI in CINs of HIV-negative patients was 6. If we considered 6 as a threshold, we noted that in HIV-positive patients, mean values of AI in CIN1 and CIN2/CIN3 below this threshold were still very significantly higher than the corresponding values in HIV-negative patients (Table 1).

In HIV-negative as in HIV-positive patients, AI augmented in function of the severity of the lesions. Thus, in both groups considered in their whole, AI was significantly lower in condylomas acuminata than in CINs. However, the difference in AI between CIN1 and CIN2/CIN3 reached almost statistical significance in HIV-positive patients only (P < 0.06; Table 1). In the restricted populations showing both CIN1 and CIN2/CIN3 in the two groups, the difference in AI between CIN1 and CIN2/CIN3 was almost significant.

Table 1. AIs in normal and pathologic cervical mucosa according to the immune status of the woman

<table>
<thead>
<tr>
<th>Cervical tissues</th>
<th>HIV-negative women</th>
<th>HIV-positive women</th>
<th>Statistics, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa in healthy women</td>
<td>0.83 ± 0.16 (n = 12) (0.17-1.95)</td>
<td>1.13 ± 0.22 (n = 7) (0.30-2.10)</td>
<td>NS, &lt;0.014</td>
</tr>
<tr>
<td>Condyloma acuminata with nononcogenic HPV</td>
<td>0.70 ± 0.14 (n = 6) (0.21-1.11)</td>
<td>4.76 ± 0.63 (n = 21) (1.11-12.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CIN with oncogenic HPV</td>
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<tr>
<td>CIN 1</td>
<td>1.17 ± 0.17 (n = 23) (0.17-3.60)</td>
<td>6.63 ± 0.72 (n = 24) (0.11-12.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CIN 2/CIN 3</td>
<td>1.67 ± 0.26 (n = 22) (0.63-6.0)</td>
<td>7.10 ± 0.84 (n = 17) (0.42-17.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Patients having two types of CIN with oncogenic HPV</td>
<td></td>
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</tr>
<tr>
<td>CIN 1</td>
<td>1.09 ± 0.23 (n = 16)</td>
<td>4.38 ± 0.70 (n = 17)</td>
<td>0.0003</td>
</tr>
<tr>
<td>CIN 2/CIN 3</td>
<td>1.62 ± 0.20 (n = 16)</td>
<td>7.10 ± 0.84 (n = 17)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AI threshold</td>
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</tr>
<tr>
<td>CIN 1, AI ≤ 6</td>
<td>1.17 ± 0.17 (n = 23)</td>
<td>3.52 ± 0.42 (n = 16)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CIN 2/CIN 3, AI ≤ 6</td>
<td>1.67 ± 0.26 (n = 22)</td>
<td>3.56 ± 0.54 (n = 12)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

NOTE: Values are mean ± 1 SE. The number of lesions examined and the range of values for the AI are indicated between parentheses. One HIV-positive woman exhibited three different condylomas acuminata on the biopsy. The highest value of AI in CIN(s) from HIV-negative women, corresponding to 6, was used as a threshold (see Fig. 2).

*Significantly different from the AI in CIN1 and CIN2/CIN3 in the same group of patients (P < 0.0003 to P < 0.0001).

1 Significantly different from corresponding values in HIV-negative patients (Mann and Whitney U test).

2 Significantly different from the AI in healthy women (P < 0.005) and from that in condyloma acuminata in HIV-negative patients (P < 0.001).

3 Significantly different from CIN1 values in the same HIV-positive women (P < 0.002; Student’s paired t test).
CINs. When using an antibody against human active caspase-3, located essentially in the cytoplasm of the surface cell layers of signal (Fig. 3E). Caspase-3 immunoreactivity was clear but represented in CIN(s) of HIV-positive women with a nuclear and and CIN2/3 (Fig. 3D). Active caspase-9 was also weakly observed in a significant number of epithelial cells in CIN1 and especially nuclear immunohistochemical signal was mucosa neighboring cervical lesions (Fig. 3C), a cytoplasmic whereas caspase-8 was weakly expressed in the subnormal especially in high-grade lesions. Thus, in HIV-positive patients, to these caspases was enhanced in the CINs from HIV-positive ing high AI. Generally, these lesions exhibited a clear-cut in the normal mucosa of healthy women (data not shown). in the HIV-negative group ($P < 0.08$) and very significant in the HIV-positive group ($P < 0.002$; Table 1).

There was no relationship between AI and either the type of oncogenic HPV or the serum CD4 T lymphocytes count per mL. Ki-67 expression did not roughly differ between HIV-negative and HIV-positive populations. The labeling was located in the parabasal epithelial cells in the normal mucosa, extending to the upper cell layers with the severity of lesions. It must be pointed out that, in the normal mucosa neighboring the Clin of HIV-positive women, AI and Ki-67 labeling were low as in the normal mucosa of healthy women (data not shown).

**Increased expression of caspases in cervical lesions from HIV-infected patients.** We examined the expression of several caspases in normal mucosa and cervical lesions from several HIV-negative and HIV-positive women, some of them presenting high AI. Generally, these lesions exhibited a clear-cut immunoreactivity to investigated caspases, except for caspase-2, which gave no signal in all samples tested. Caspase-8 (proform), caspase-3 (proform), active caspase-9, and active caspase-3 were expressed in the normal mucosa and Clin from HIV-negative patients but in rare to few cells scattered in the mucosa (Fig. 3A-B). The number of immunopositive cells to these caspases was enhanced in the Clin from HIV-positive patients by comparison with those from HIV-negative ones, especially in high-grade lesions. Thus, in HIV-positive patients, whereas caspase-8 was weakly expressed in the subnormal mucosa neighboring cervical lesions (Fig. 3C), a cytoplasmic and especially nuclear immunohistochemical signal was observed in a significant number of epithelial cells in Clin1 and CN2/3 (Fig. 3D). Active caspase-9 was also well represented in Clin(s) of HIV-positive women with a nuclear signal (Fig. 3E). Caspase-3 immunoreactivity was clear but located essentially in the cytoplasm of the surface cell layers of Clins. When using an antibody against human active caspase-3, a strong cytoplasmic and nuclear signal was confirmed in the same areas of tissue samples (Fig. 3F).

**Immunohistochemical detection of Langerhans’ cells in normal and pathologic epithelium.** Langerhans’ cells act as antigen-presenting cells and induce a T-dependent immune response. They have also been reported to be involved in the mechanisms of apoptosis in the squamous genital epithelium (1). To analyze the possible interaction of these cells with the AIs in normal and pathologic cervical mucosa, we identified them by their positive reaction with the CD1a antibody. In the normal mucosa from healthy women, condyloma acuminata and Clin with oncogenic HPV in HIV-negative patients, Langerhans’ cells were scattered throughout the mucosa, some of them being located near the surface (Fig. 4A-C). They exhibited long cytoplasmic processes in contact to epithelial cells sometimes far from them. Langerhans’ cells were rare or absent in high-grade lesions of HIV-positive patients (Fig. 4D). In addition, they showed morphologic alterations, especially shortened dendritic processes. With regard to Langerhans’ cells densities (number of cells per mm of mucosa), there was no statistical difference between values in normal mucosa ($21 \pm 3$), condyloma acuminata from HIV-negative women ($15.2 \pm 3.9$) or from HIV-positive women ($13.5 \pm 2.6$), and Clin1 or CN2/CN3 from HIV-negative women ($18 \pm 2$ and $14.9 \pm 1.3$, respectively). However, there was a significant decrease in Langerhans’ cell densities as compared with normal values ($P < 0.0001$) in Clin1 and CN2/CN3 from HIV-positive women ($5.8 \pm 0.7$ and $2.9 \pm 0.9$, respectively). Moreover, within the HIV-positive group, Langerhans’ cell

![Image](https://example.com/image.png)

**Fig. 1.** A–E, illustrations of TUNEL labeled cells in human cervical mucosae. Arrows, some apoptotic nuclei in those mucosae. A, normal cervical mucosa from an healthy woman showing one cell with an apoptotic nucleus. Right, higher magnification of the apoptotic cell located in the middle third of the epithelial thickness. B, Clin of grade 2 with oncogenic HPV from one HIV-negative woman. Note the rare labeled apoptotic cells at the surface of mucosa. C, Clin of grade 2 with oncogenic HPV from one HIV-infected woman displaying numerous apoptotic nuclei gathered in foci. Right, detail of apoptotic cells in the upper third epithelial layers. D, Clin of grade 2 from another HIV-infected woman. Note the numerous apoptotic nuclei at the surface. Inset, detail of apoptotic nuclei. Bar, 50 μm.

![Image](https://example.com/image.png)

**Fig. 2.** Diagram showing the profile of distribution of individual values of the AI in the normal cervical mucosa and the different groups of cervical lesions from HIV-infected and noninfected women. Horizontal line, highest AI individual values, 6, attained in cervical lesions from HIV-negative women and considered a threshold.
densities were significantly lower in CIN2/CIN3 than in CIN1 \((P < 0.02)\). Significant decreases in Langerhans' cell densities were also found in CINs of HIV-positive women as compared with the corresponding values in CINs of HIV-negative women \((P < 0.0001)\).

**Apoptotic indices are correlated with Langerhans' cell densities in normal and pathologic epithelium.** We also examined the correlation between the Langerhans' cell density and the AI. There was a strong linear inverse correlation between mean values of Langerhans' cell and AI in the normal mucosa and the different groups of lesions \((r = -0.88, P < 0.002)\). Similarly, individual values of the two variables in each CIN1 and CIN2/ CIN3 of HIV-positive women were inversely correlated \((r = -0.49 \text{ for both linear and Spearman rank correlations, } P = 0.0007)\). Only a Spearman rank correlation between individual Langerhans' cell density and AI was found in CINs of HIV-negative women \((r' = -0.45, P < 0.003)\). The two types of correlation existed also for the whole of individual values of Langerhans' cell and AI in all CINs from HIV-negative and HIV-positive women \((r = -0.59 \text{ and } r' = -0.75, P < 0.0001)\).

**Expression of other markers.** Cervical samples displayed no Fas immunostaining. There was only a weak and inconstant Fas ligand signal on cell membranes, whatever the grade of dysplasia. Protein p53 was not expressed in oncogenic HPV lesions confirming that E6-HPV protein eliminates the p53 detection \((29)\). Bcl2 was present in the basal epithelial layers as previously described \((30)\) but only in few cells and with a weak staining.

**Discussion**

Little is known about the natural history of HPV infection in HIV-positive women and mechanisms by which HPV and HIV induce carcinoma \((31)\). HIV infection has been shown to increase the prevalence of HPV-related intraepithelial lesions in
anogenital squamous epithelium (26, 32, 33) and to augment the relative risk of cervical and anal carcinoma (11, 12). However, we and others have pointed out that relatively few squamous invasive cancers develop in tritherapy untreated HIV-infected population despite the high incidence of high-grade CIN (22). Two possibilities may account for this observation (i) the diagnosis was established early and HPV lesions fast destroyed and/or (ii) an imbalance may exist between cell proliferation and apoptosis in favor of apoptosis. We decided in this study to explore this second hypothesis.

First of all, several reports have investigated the apoptosis in the uterine cervical mucosa of HIV-negative women in normal and pathologic conditions. In these reports, CIN of grade 2 has been grouped either with CIN of grade 1 or with CIN of grade 3, depending on authors, as indicated in Table 2. With the exception of a single work which described that increasing cervical atypicality is associated with a decrease in apoptosis (34), all authors found that the AI is very low in the normal cervical epithelium and increases with the progression of cervical neoplasia through CIN to carcinoma (5–9). Our present results confirm this second statement. Moreover, our estimation of AIs in the normal mucosa, condyloma and CIN from HIV-negative patients were in the range of those previously reported (Table 2). Some authors have underlined the lack of correlation between the AI and the presence of HPV in lesions (5–7). In addition, it was found that in

Table 2. AIs in normal cervical mucosa and in cervical intraepithelial neoplasia of women noninfected with HIV

<table>
<thead>
<tr>
<th>Cervical samples</th>
<th>Shogi et al. (5)</th>
<th>Isacson et al. (6)</th>
<th>Duttagupta et al. (7)</th>
<th>Lee et al. (8)</th>
<th>Zanotti et al. (9)</th>
<th>Our present data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>0</td>
<td>0.23 ± 0.7</td>
<td>0.13 ± 0.02</td>
<td>0.83 ± 0.16</td>
<td>0.70 ± 0.14</td>
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<td>Condyloma (HPV 6 and HPV 11)</td>
<td>0.13 ± 0.05</td>
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<tr>
<td>CIN 1</td>
<td>0.17 ± 0.04*</td>
<td>2.68 ± 0.42</td>
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<td></td>
<td>1.17 ± 0.17*</td>
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<tr>
<td>CIN 1/CIN 2</td>
<td>0.26 ± 0.08</td>
<td></td>
<td></td>
<td></td>
<td>1.67 ± 0.26*</td>
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<tr>
<td>CIN 2/CIN 3</td>
<td>0.98 ± 0.27</td>
<td>3.24 ± 0.44</td>
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<td>0.4 ± 0.5</td>
<td></td>
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<tr>
<td>CIN 3</td>
<td>0.82 ± 0.17</td>
<td>1.25 ± 0.07</td>
<td>1.3 ± 1.1</td>
<td>1.76 ± 0.38</td>
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</tr>
<tr>
<td>Squamous carcinoma</td>
<td>1.81 ± 0.39†</td>
<td>1.69 ± 0.09</td>
<td>4.1 ± 2.3</td>
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<tr>
<td></td>
<td>to 4.65 ± 0.44†</td>
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</tbody>
</table>

NOTE: Mean ± SE. TUNEL method except in situ end labeling method for ref. 9.
*Lesions without oncogenic HPV.
†Lesions with oncogenic HPV.
*Estimated in 12 lesions.
†Microinvasive carcinoma.
†Invasive squamous carcinoma.
HPV-infected lesions of similar grade, the type(s) of HPV, nononcogenic or oncogenic, does not significantly affect AIs (6). Consistent with this finding, because all our patients presented with HPV lesions, we also noted that there was no significant difference between the AI in condyloma acuminata infected with nononcogenic HPV and that in CIN of grade 1 infected with oncogenic HPV. However, few condyloma acuminata specimens were examined with respect to the number of CIN studied. It must be pointed out that it is difficult to obtain condyloma acuminata samples from the cervix because they were relatively rare in that location and they were in most cases destroyed by laser. With regard to caspases which are apoptosis-related proteins, contradictory results have been reported in cervical lesions about caspase-3. For some authors, the percentages of cases with expression of caspase-3 was significantly higher in CIN of high grade than in CIN of low grade (10) as was the AI, whereas others found that active caspase-3 expression slightly decreased with increasing dysplasia, being restricted in few cells in invasive carcinoma (9). In the present study, we have not quantified the cervical epithelial cells expressing caspases in HIV-negative patients, but the apparent number of cells positive to caspase-8, active caspase-9 or active caspase-3 was compatible with that of TUNEL-positive cells in a given lesion.

Second, herein, we report for the first time that epithelial cell apoptosis in cervical lesions is dramatically enhanced in HIV-infected women, the AI increasing with the severity of the lesion. Indeed, the increase in AI was only minor (1.6-fold) in condylomas acuminata with nononcogenic HPV but was strong (4-fold) in CINs with oncogenic HPV by comparison with the same type of lesions in HIV-negative women. This indicates that likely, the presence of oncogenic HPV amplified the influence of HIV infection. The higher AI in HIV-infected patients was accompanied by increased numbers of cells expressing caspase-8, active caspase-9, and active caspase-3. We also noted that, in the same tissue samples, TUNEL labeling and caspase expression were localized in the same areas (see for instance Figs. 1D and 3D-F). Despite evidence of caspase-8 proform expression in lesions from HIV-positive women, we had not the opportunity to study active caspase-8 because the commercial antisera was not available. By contrast, our data showed that caspase-9 was activated in these lesions and at its turn must probably activate caspase-3. These results suggest that apoptosis is mediated by the mitochondrial pathway (20). Apoptosis is known to highly occur in CD4+ T lymphocytes from HIV-infected patients (13–16). Recently, apoptosis was found to be increased in cells of different tissues in HIV-associated pathologies (17–21). Apoptosis in these cells may be promoted by the action of coat proteins linked to the HIV, among them Tat-1 (15, 20), Vpr (14, 18), and gp120 (19, 21). Our results thus added a new element (i.e., epithelial cell of the pathologic cervix), to the list of cells affected by the high incidence of apoptosis in HIV disease. The mean levels of AI we found in the CINs of low and high grade from HIV-positive patients showed a 5.7- to 7.9-fold increase over that in normal epithelium. This estimation is consistent with the mean number of active caspase-3-positive neurons in HIV-associated neurodegeneration (6.0 ± 0.5 versus 1.0 ± 0.5 in controls) showing a 6-fold increase over normal neurons (19).

It is known that HIV does not infect epithelial squamous cells per se but is present in small amount in Langerhans’ cells of squamous tissues notably in cervical mucosa (35–37). This property is currently explored in inactivated whole virus-pulsed dendritic cell vaccines and seemed promising to control diseases caused by HIV (38). Apoptotic cell elimination may be assured by usual phagocytes such as macrophages but also by other cells like immature dendritic cells among them Langerhans’ cells (1–3). In HIV infection, Langerhans’ cells displayed morphologic changes and reduced number (23–25, 39–41) especially in the submucosa of the female genital tract (41). Herein, we confirmed that HIV infection was associated with a decrease in Langerhans’ cell number in the squamous intraepithelial cervical lesions and have shown that they were inversely correlated with AIs. The alteration of their dendritic processes suggests that the function of Langerhans’ cells, notably antigen presentation and apoptotic cell elimination, may be impaired.

Several mechanisms may be implicated in the high incidence of apoptosis in cervical samples of HIV-infected women and consequently the relatively low incidence of invasive carcinoma. We suggest that three of these mechanisms could be related, alone or together, to HIV-infected Langerhans’ cells. Because the latter are involved in the elimination of apoptotic cells in squamous genital mucosa, an apoptotic cell accumulation, independent of the rate of the apoptotic process, may be favored by (i) the diminution of the Langerhans’ cell number; (ii) the diminution of the phagocytic process per se. Indeed, it has been proven that HIV-1 Tat inhibits the engulfment of apoptotic bodies by dendritic cells (42). (iii) Alternatively, a real acceleration of the apoptotic process may occur in epithelial cells, likely due to the cytotoxic action of proteins presented by HIV-infected CD4+ T cells and Langerhans’ cells (37, 43–45). Another mechanism could be linked to the hepatocyte growth factor. Indeed, we have recently shown that the proteins of hepatocyte growth factor and its receptor c-Met are overexpressed in the CINs of HIV-infected women (27). Hepatocyte growth factor exerts through its receptor a proapoptotic effect on some tissues and under certain conditions (46–49). This is the case for instance in ovarian carcinoma (49). Thus, if this effect exists in the cervix, hepatocyte growth factor may contribute to promote apoptosis in the intraepithelial lesions. Evidently, future studies must be carried out in CINs from tritherapy-treated HIV-infected subjects to examine whether apoptosis in epithelial cells is reduced to the normal level as this has been shown for CD4+ T lymphocytes (16).

In conclusion, the present study provides evidence for the first time that in HIV-infected women the incidence of apoptosis is strongly enhanced in intraepithelial lesions of the cervix. This was confirmed by higher active caspase-9 and active caspase-3 expression. This suggests at least a mitochondrial-mediated pathway conducing to apoptosis. This study highlights also the potential role that Langerhans’ cells could play in this process.

Acknowledgments

We thank Professor Emile Daraï for his cooperation; Elisabeth Soustre, Isabelle Prevost, Michelle Saadoun, Laurence Lemen, and Aïda Debue for invaluable technical assistance; Vincent Gramond and Laure Guillaudeau for help with illustrations; and Jean Pierre Laigneau for help in graphic.
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