Hepatocyte Growth Factor and c-Met in Cervical Intraepithelial Neoplasia: Overexpression of Proteins Associated with Oncogenic Human Papillomavirus and Human Immunodeficiency Virus\(^1\)

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ABSTRACT

Purpose: High prevalence of squamous cervical intraepithelial neoplasia (CIN) linked to oncogenic human papillomavirus (HPV) exists in HIV-infected women. Hepatocyte growth factor (HGF) and its receptor, c-Met, promote cell proliferation and are involved in tumor progression. Nothing is yet known about their expression in low- and high-grade CIN. Therefore, the expression, localization, and behavior of HGF and c-Met in normal and dysplastic cervical epithelium were investigated.

Experimental Design: We studied normal cervical mucosa from 10 healthy women, and low- and high-grade cervical lesions, uninfected (condyloma acuminata) or infected with oncogenic HPVs, from 40 HIV-negative and 48 HIV-positive women, using in situ molecular techniques, immunocytochemistry and morphoquantitative methods.

Results: In 154 oncogenic HPV-infected CIN encountered in biopsy samples, the total number of epithelial cell layers increased significantly during lesion progression. This number was significantly higher in HIV-positive than in HIV-negative women for CIN1 and CIN2 (\(P < 0.025\) to \(P < 0.001\)). In HIV-negative women, the number and percentage of HGF and c-Met immunostained cell layers, and the intensity of immunostaining were enhanced in oncogenic HPV-infected lesions as compared with normal mucosa and condyloma acuminata. The latter parameters were significantly higher in tissues of HIV-positive women (oncogenic HPV-infected CIN1 and CIN2, normal-appearing mucosa contiguous to CIN, condyloma acuminata) than in the corresponding tissues of HIV-negative women (\(P < 0.025\) to \(P < 0.0001\)).

Conclusions: Overexpression of HGF/c-Met complex strongly correlates with oncogenic HPV and HIV infection. This overexpressed complex may stimulate cell proliferation in condyloma acuminata and participate in tumor progression in oncogenic HPV-infected lesions.

INTRODUCTION

The multistage nature of carcinogenesis in the cervical epithelium makes it possible to analyze the successive events that intervene, from the disturbance of cell proliferation and/or differentiation in squamous epithelium, via low- and high-grade CIN,\(^3\) also called SIL, to invasive carcinoma. HPV has been implicated in the development of genital intraepithelial neoplasia (1). All genital condylomas, and most intraepithelial neoplasia and invasive cervical cancers contain HPV DNAs (2, 3). HPV types 16, 18, 31, and 33 are most commonly found in cervical, vaginal, or vulvar neoplasia, whereas types 6 and 11 are linked to condyloma acuminata or regressive dysplasia (4). HIV is another widespread infection, which also per se increases the risk of carcinogenesis (5, 6). Patients with HIV infection have been shown to present a high prevalence of both cervical HPV infection and CIN. Little is known about the natural history of HPV infection in HIV-positive women, and mechanisms by which HPV and HIV induce carcinoma (7).

Aberrant expression of gene products of several growth factors and/or their receptors, such as HGF and its receptor, c-Met, may be associated with genetic alterations in epithelial cells which, in turn, may be linked to the carcinogenesis process. HGF is a glycoprotein consisting of a heavy \(\alpha\) chain and a light \(\beta\) chain, held together by a disulfide bond. The two chains have molecular masses of 70 and 35 kDa, respectively. HGF is thought to be produced principally by mesenchymal cells, although mRNA and protein have sometimes been detected in epithelia (8–11). The HGF receptor, encoded by the c-met proto-oncogene, contains a 145-kDa transmembrane \(\beta\) chain with tyrosine kinase activity. C-Met activation by its ligand

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\(^1\) The abbreviations used are: CIN, cervical intraepithelial neoplasia; SIL, squamous intraepithelial lesion; CMV, cytomegalovirus; HGF, hepatocyte growth factor; HPV, human papillomavirus; ISH, in situ hybridization; RT-PCR, reverse transcription-PCR; HSV, herpes simplex virus.
leads to pleiotropic biological effects on various epithelial target cells, including the induction of cell proliferation, morphogenetic transformation, cell motility, and invasiveness, under both normal and pathological conditions (12, 13). The HGF/c-Met complex is thought to contribute to the progression of numerous epithelial cancers (14–16).

In the female genital tract, the presence of HGF protein has been mentioned in normal endometrial, vaginal, and ovarian epithelial cells (8). Increased secretion of HGF by eutopic endometrial cells has been found in women with endometriosis (17). The receptor c-Met has been reported to be present in various amounts in the normal endometrium and ovarian epithelium (18–21), and overexpressed in ovarian carcinomas (20, 21). Moreover, in vitro, HGF is able to stimulate the proliferation and migration of human endometrial and ovarian carcinoma cells (22–24), as well as the scattering and morphogenesis of human cervical carcinoma cells (25). However, nothing is yet known about the expression of HGF and c-Met in squamous cervical intraepithelial lesions, particularly those linked to oncogenic HPV(s) in HIV-negative and HIV-positive populations.

Therefore, we were prompted, using immunohistochemistry, morphoquantitative methods, and in situ RT-PCR, to study the expression, localization, and behavior of HGF and c-Met in normal squamous cervical epithelium, and in low- (including acuminata condyloma) and high-grade cervical intraepithelial lesions, linked or not to oncogenic HPV(s) infection, in women with and without HIV infection. We then tried to analyze the effects on these variables of HPV and the HIV-positive status.

MATERIALS AND METHODS

Patients and Tissues. Eighty-one specimens of squamous cervical lesions infected with oncogenic HPV(s) were obtained from 77 women treated at the Bichat-Claude Bernard Hospital, Paris, France; 34 were HIV-negative (8500 consecutive HIV-negative women were tested, giving a prevalence of oncogenic HPV infection of 0.4%) and 43 were HIV-positive (71 tested, giving a prevalence of oncogenic HPV infection of 63%). We used in situ molecular techniques, immunohistochemistry, and/or viral cultures to check that these women had no other significant viral infections (HSV, EBV, or CMV). HIV status was confirmed by Western immunoblotting. Twenty-one of the HIV-positive patients were immunocompromised (serum CD4 T lymphocytes <200/ml). The characteristics of these patients are summarized in Table 1. We also studied condyloma acuminata samples infected with nononcogenic HPVs 6 and 11 isolated from a small cohort of patients (6 HIV-negative and 5 HIV-positive), as well as biopsies of normal cervical mucosa removed from 10 healthy women who had undergone hysterectomy for leiomyoma. All of the patients gave consent for biopsies before inclusion. The study was approved by the Human Research Committee of the Bichat-Claude Bernard Hospital.

All of the HIV-positive patients underwent colposcopy and systematic biopsies, whereas HIV-negative patients with condylomas were selected on the basis of pap-smear examination followed by colposcopy and biopsies. For all of the patients included in the study, microbiologic tests for Chlamydia, Gonococcus, Mycoplasma, and Mycobacterium, and serological tests for syphilis were negative. All of the 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 histological diagnosis, and periodic-acid Schiff, Zielh, and Grocott reagents for the detection of microbiological agents. Subsequent sections were used for the detection of HPV and other viruses, and for HGF and c-Met immunostainings.

Histological Analysis. Flat condyloma has an appearance of “glycogenic acanthosis.” It is characterized by dysplastic areas of two grades: low-grade and high-grade dysplasia. CIN of low grade, or CIN1, was defined as thickening of the squamous epithelium, moderately atypical cells in the lower squamous layers, with irregular nuclei and hyperchromatism, but with no atypical mitosis, and differences between basal and suprabasal cells with delayed glycogenic maturation. Cervical intraepithelial neoplasia of high-grade combined CIN of grades 2 (moderate dysplasia, CIN2) and 3 (severe dysplasia and intraepithelial cancer, CIN3). It was defined as the loss of normal stratification of the cells, defect in cell maturation, severe architectural disruption, and the presence of atypical cytoplasm and nuclear features throughout the epithelium: nuclear pleomorphism and hyperchromatism, atypical and numerous mitosis, and cytoplasmic basophilia with or without dyskeratosis (26).

Condyloma acuminata is considered as low-grade SIL (CIN1). It was diagnosed based on standard histological criteria: hyperpapillomatosis 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 parakeratosis or orthokeratosis was associated in some cases.

In Situ Molecular Techniques for Detection of HPVs. ISH and PCR-ISH were used for HPV screening and typing, with PCR-ISH performed only for samples that appeared HPV-negative by ISH alone. For ISH, biotinylated and FITC-labeled commercial genomic DNA probes were purchased from Argène (Varhiles, France) and Dakopatt (Glostrup, Denmark). For PCR-ISH, primers MY11 and MY09 (Perkin-Elmer, Norwalk, CT), 20 nucleotides long, corresponding to part of the L1 major protein of the viral capsid, were used. ISH and PCR-ISH were performed according to our protocol published previously (see Ref. 27 for detail). In some cases, for biotinylated probes, the

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**Table 1** Characteristics of HIV-negative and HIV-positive women with oncogenic HPV(s) in cervical lesions

<table>
<thead>
<tr>
<th></th>
<th>HIV-negative women</th>
<th>HIV-positive women</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>34</td>
<td>43</td>
</tr>
<tr>
<td>Age (yrs: median, range)</td>
<td>31 (17.5–67.5)</td>
<td>31.5 (21–46.5)</td>
</tr>
<tr>
<td>Ethnic origins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>32 (94.1%)</td>
<td>29 (67.5%)</td>
</tr>
<tr>
<td>African</td>
<td>1 (2.9%)</td>
<td>13 (30.2%)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (2.9%)</td>
<td>1 (2.3%)</td>
</tr>
<tr>
<td>Oncogenic HPV types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPV 16</td>
<td>18 (52.9%)</td>
<td>25 (58.1%)</td>
</tr>
<tr>
<td>HPV 18</td>
<td>8 (23.5%)</td>
<td>9 (20.9%)</td>
</tr>
<tr>
<td>HPV 31</td>
<td>6 (17.4%)</td>
<td>6 (14%)</td>
</tr>
<tr>
<td>HPV 33</td>
<td>2 (5.9%)</td>
<td>9 (20.9%)</td>
</tr>
</tbody>
</table>

*a* Indian.  
*b* West Indian.
signal was amplified with the biotinyl-tyramide complex and the diaminobenzidine chromogen (Genpoint kit; Dakopatt). The specificity of ISH was assessed by omitting probes, and that of the diaminobenzidine chromogen (Genpoint kit; Dakopatt). The signal was amplified with the biotinyl-tyramide complex and revealed by diaminobenzidine. Hematoxylin nuclei counterstaining. The signal is very strong in koilocytes in (a; arrows), and almost the entire thickness of CIN3 (b). Bar = 50 μm.

Evaluation of Immunostaining. Sections were examined under a Leitz Orthoplan microscope and tissue reactions assessed in two ways. First, the presence and intensity of HGF or c-Met immunostaining in normal mucosa and each lesion encountered all along tissue samples were evaluated independently by two researchers (F. W. and T. L.) with a semiquantitative method: 0, negative; (±), just detectable; ±, weak; +, moderate; ++, strong; ++++, intense. The classification of staining was reassessed after a second examination 1 month later to ensure that the data obtained were reliable. In most cases, the two evaluations were concordant. However, in rare cases, specimens were definitively classified after discussion between the two observers and a third examination.

Second, we used a morphoquantitative method. For the normal mucosa, condyloma acuminata, and each grade of CIN encountered in a given tissue specimen, the two observers counted the number of component epithelial cell layers, and the number of cell layers displaying immunostaining for c-Met and HGF. We then calculated the percentage of the labeled cell layers over the total number of cell layers in the normal mucosa or lesions. Counts were performed at a magnification of ×400, and <5% interindividual variation in counts was observed. The staining of normal-appearing tissue adjacent to CIN lesions, within the same tissue section, was also evaluated whenever possible.

In Situ Molecular Technique for HGF and c-Met mRNA Detection. We used the in situ RT-PCR technique with the following oligonucleotide primers synthesized and then 5’ biotinylated by Genset (Paris, France): for c-Met, sense primer was 5’-TACTTGTGTGCAAGGAGAAGACTCCTA-3’; and antisense primer was 5’-GGGACCAAGCCTCTGGT-TCTGATGC-3’; and for HGF, sense primer was 5’-CAGCGTT-TGGGATTCTCAGTAT-3’ and antisense primer was 5’-CCTATGTGTTTCTGTGTTCGA-3’ (9). Reverse transcription secondary antiamouse or antigoat IgG diluted 1:200. and finally with the Elite complex diluted 1:100 (kit Elite PK6100 Vectorstain; Vector Laboratories, Burlingame, CA). Immunoreactivity was revealed by diaminobenzidine, and nuclei were counterstained with hematoxylin. Immunostaining specificity was checked either by overnight preincubation of the primary HGF antibody with 20 μg HGF (Sigma) per ml of diluted antiserum before immunoreaction or by omission of the primary anti-c-Met antibody.

Table 2 Number, grade, and cell layer component of CIN, related to oncogenic HPV(s), in HIV-negative and HIV-positive women

<table>
<thead>
<tr>
<th></th>
<th>HIV-negative women</th>
<th>HIV-positive women</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>CIN 1</td>
<td>25</td>
<td>34</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>CIN 2</td>
<td>28</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>CIN 3</td>
<td>12</td>
<td>19</td>
<td></td>
</tr>
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</table>

a In HIV-negative women, P < 0.03 vs. CIN 1.
b In HIV-positive women, P = 0.05 vs. CIN 1.
c In HIV-negative women, P < 0.003 vs. CIN 1 and P < 0.04 vs. CIN 2.
d In HIV-positive women, P = 0.01 vs. CIN 1.

Figure 1 Detection in HIV-infected patients of HPV 11 in one condyloma acuminata (a) and HPV 16 in CIN3 (b) by ISH with biotinylated probes. The signal was amplified with the biotinyl-tyramide complex and revealed by diaminobenzidine. Hematoxylin nuclei counterstaining. The signal is very strong in koilocytes in (a; arrows), and almost the entire thickness of CIN3 (b). Bar = 50 μm.
procedure was performed with the RT-PCR kit provided by Perkin-Elmer (Norwalk, CT) according to the manufacturer’s recommendation. Then, the PCR procedure with the labeled primers consisted of 25 amplification cycles. The amplicons obtained were thus directly biotinylated, and their detection in tissues was made using amplification of the signal with the biotinyl-tyramide complex and the diaminobenzidine chromogen. Negative controls were obtained in performing the reaction.

**Fig. 2** C-Met immunostaining in cervical lesions infected with oncogenic HPV(s) from 3 HIV-positive women. Photographs show all thickness of epithelial lesions. Note that, within a given specimen, both the intensity of the immunoreaction and its distribution increased as a function of CIN severity, with the involvement of increasing numbers of cell layers in the mucosa. Patient 1 (a), normal mucosa near oncogenic HPV lesions with slight signal in the basal cell layers (arrow), compare with b, CIN of grade 2; patient 2 (c), CIN of grade 1, compare with d, CIN of grade 2; patient 3 (e), CIN of grade 2, compare with f, CIN of grade 3. Note the strong signal in the superficial layers (arrow) in e and throughout the epithelium in f. Bar = 50 μm.
Fig. 3  C-Met immunostaining (left) and HGF immunostaining (right) in cervical lesions infected by oncogenic HPV(s) from 1 HIV-negative woman (a and b) and 4 HIV-positive women (c–f). Photographs of the entire thickness of the epithelial lesions were taken for the patients who gave the strongest reactions to antibodies. Note the weak immunostaining of c-Met throughout the CIN of grade 2, more evident near the surface (arrow), in the HIV-negative patient and the greater intensity of the immunoreaction for c-Met and HGF in HIV-positive patients, even in lesions of lower grade than that from the HIV-negative patient. a and b, patient 1, CIN of grade 2; c, patient 2, CIN of grade 1; d, patient 3, CIN of grade 1; e, patient 4, CIN of grade 2; f, patient 5, CIN of grade 3. Note also the immunostaining of koilocytes in c and e (arrows), and the variable location of the strongest intensity either in the upper half (c) or the lower half of epithelium (d). Hematoxylin nuclei counterstaining. Bar = 50 μm (a, b, and f, same magnification; c–e, same magnification).
with omission of the Taq polymerase and primers. It was also checked that, after simple incubation of slides in buffer, the biotinyl-tyramide-diaminobenzidine procedure by itself did not reveal any false-positive signal in tissues.

Statistical Analysis. Quantitative data were expressed as means ± 1 SEM. Differences between two groups were evaluated with the Student t test or the Mann-Whitney U test when-ever relevant. P values < 0.05 (two-tailed tests) were considered to be statistically significant.

RESULTS

Typing of HPV in Cervical Lesions. ISH and PCR-ISH allowed the identification of HPV 6 and 11 in condylo-mata, and HPV 16, 18, 31, and 33 in flat condylomas with dysplasia, in HIV-negative and HIV-positive women (Fig. 1). The percentages of women with oncogenic HPV lesions caused by types 16, 18, and 31 were similar in the two groups, regardless of HIV status, with HPV 16 the most frequently represented (in 53–58% of patients; Table 1). However, the frequency of HPV 33 differed between HIV-negative and HIV-positive women, but we have checked that this difference did not affect the results, as no relationship to the level of HGF or c-Met expression was observed. In HIV-negative patients, all of the lesions were HPV monoviral, whereas 3 of the HIV-positive patients had biviral lesions, and 2 others had triviral lesions.

Immunostaining, ISH, or viral cultures provided no evidence for the presence of CMV, EBV, or HSV in any of these lesions.

In Patients Infected with Oncogenic HPV(s), the Intensity of Immunostaining for HGF and c-Met Increases with the Severity of CIN. A total of 154 squamous cervical lesions containing oncogenic HPVs were analyzed. All three grades of CIN were observed in HIV-positive and in HIV-negative women (Table 2). It was found that the cell layer component increased with the severity of CIN in the two populations (Table 2). Moreover, the absolute number of cell layers for CIN of grade 1 and CIN of grade 2 was significantly higher in HIV-positive patients than in HIV-negative patients (Table 2).

HGF and c-Met proteins, when present, could be immuno-detected throughout the entire thickness of the pathological cervical mucosa but were preferentially distributed in the germinative and suprabasal layers, koilocytes, and sometimes superficial layers (Figs. 2 and 3). Immunostaining for the two proteins was also seen in the glandular cells of the endocervix. These signals were specific as they were abolished by incubation of the HGF antibody with its homologous antigen before immunoreaction or omission of the c-Met antibody.

Histopathological examination showed that the amount of HGF and c-Met gradually increased with the severity of the CIN containing oncogenic HPV(s). This was evident in both the

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Number and percentage of cell layers immunoreactive to c-Met in lesions infected with oncogenic HPV(s), according to the grade of CIN and the immune status of the woman</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN 1</td>
<td>No. immunoreactive cell layers</td>
</tr>
<tr>
<td>10.7 ± 1.3 (25)</td>
<td>15.3 ± 1.4 (34)</td>
</tr>
<tr>
<td>12.2 ± 1.4 (26)</td>
<td>21.1 ± 1.5% (32)</td>
</tr>
<tr>
<td>16.9 ± 3.9 (11)</td>
<td>26.2 ± 3.6’s (19)</td>
</tr>
<tr>
<td>CIN 2</td>
<td></td>
</tr>
<tr>
<td>61.3 ± 6.4 (25)</td>
<td>72.9 ± 4.6 (34)</td>
</tr>
<tr>
<td>62.2 ± 6.3 (26)</td>
<td>83.0 ± 3.6 (32)</td>
</tr>
<tr>
<td>71.2 ± 10.4 (11)</td>
<td>83.7 ± 7.0 (19)</td>
</tr>
<tr>
<td>CIN 3</td>
<td></td>
</tr>
</tbody>
</table>

* In HIV-positive women, P < 0.007 vs. CIN 1.
† In HIV-positive women, P = 0.01 vs. CIN 1.

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Number and percentage of cell layers immunoreactive to HGF in lesions infected with oncogenic HPV(s), according to the grade of CIN and the immune status of the woman</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIN 1</td>
<td>No. immunoreactive cell layers</td>
</tr>
<tr>
<td>11.2 ± 1.7 (25)</td>
<td>16.0 ± 1.3 (33)</td>
</tr>
<tr>
<td>12.3 ± 1.7 (28)</td>
<td>19.6 ± 1.4b (34)</td>
</tr>
<tr>
<td>17.5 ± 4.8 (12)</td>
<td>20.5 ± 4.2 (17)</td>
</tr>
<tr>
<td>CIN 2</td>
<td></td>
</tr>
<tr>
<td>63.3 ± 6.9 (25)</td>
<td>74.8 ± 4.0 (33)</td>
</tr>
<tr>
<td>57.7 ± 6.4 (28)</td>
<td>80.3 ± 4.0 (34)</td>
</tr>
<tr>
<td>55.9 ± 12.1 (12)</td>
<td>69.4 ± 9.2 (17)</td>
</tr>
<tr>
<td>CIN 3</td>
<td></td>
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</table>

* In HIV-positive women, P < 0.06 vs. CIN 1, not quite significant.
HIV-negative and the HIV-positive group, based on qualitative and quantitative data. Fig. 2 illustrates this finding for c-Met immunostaining in 3 HIV-positive patients, the reaction, within a given specimen, being more intense in a CIN of grade 2 than in the normal cervical mucosa near the CIN (patient 1), in a CIN of grade 2 than in a CIN of grade 1 (patient 2), and in a CIN of grade 3 than in a CIN of grade 2 (patient 3). Our quantitative data confirmed this augmentation in expression as the mean number of cell layers labeled for HGF and c-Met and, in most cases, their percentage with respect to the total cell layer population steadily increased with CIN grade in the two groups of patients (Tables 3 and 4). Thus, in HIV-negative patients, the numbers of c-Met immunostained cell layers noted in CINs of grades 2 and 3 were significantly higher than those in CIN of grade 1 (Table 3).

In Patients Infected with Oncogenic HPV(s), the HIV Seropositivity Is Associated with the Overexpression of HGF and c-Met Proteins. The HIV-positive status clearly had another remarkable effect, with HIV-positive women displaying higher levels of HGF and c-Met proteins than HIV-negative women. This was shown again by both qualitative and quantitative data, and for the two proteins.

Thus, Fig. 3 provides examples of some of the most intense immunostainings observed for c-Met and HGF in CINs from 1 HIV-negative patient and 3 HIV-positive patients. The immunostaining reaction for the two proteins dramatically increased in the HIV-positive patients. Overall, in HIV-negative women, one (3%) and four tissue specimens (12%) were totally negative for staining with anti-c-Met and anti-HGF antibodies, respectively, whereas none of the tissue specimens from HIV-positive women were entirely negative, although some areas in these specimens did not respond to antibodies. We analyzed the intensity of immunostaining for each lesion (according to the criteria defined in “Materials and Methods”) and grouped the data into three classes: class 1 (absent or just detectable), class 2 (weak to moderate), class 3 (strong to intense). Note that none of the samples from HIV-negative patients displayed class 3 immunostaining intensity and that the frequency of class 1 was lower in CIN of grade 2 from HIV-positive patients than in those from HIV-negative patients.

Fig. 4 Histograms showing the distribution profile of c-Met immunostaining intensity in low-grade CIN1 and high-grade CIN2 and CIN3 infected with oncogenic HPV, in HIV-negative women (a) and HIV-positive women (b). A semiquantitative classification was used: class 1, absent or just detectable; class 2, weak to moderate; class 3, strong to intense. Note that none of the samples from HIV-negative patients displayed class 3 immunostaining intensity and that the frequency of class 1 was lower in CIN of grade 2 from HIV-positive patients than in those from HIV-negative patients.

Fig. 5 Histograms showing the distribution profile of HGF immunostaining intensity in cervical lesions infected with oncogenic HPV(s). Same legend as for Fig. 4. Note the higher percentage of lesions with class 3 intensity and the lower percentage of lesions with class 1 intensity (particularly for CIN of grade 2) in samples from HIV-positive women than in samples from HIV-negative women.
in HIV-positive women. As for c-Met, the frequency of intensity class 1 staining was lower in HIV-positive women, particularly in CIN of grade 2.

Quantitative results confirmed that HGF and c-Met were overexpressed in the presence of HIV. Indeed, whatever the protein examined, the mean numbers and percentages of immunostained cell layers were higher in HIV-positive than in HIV-negative women, for all grades of CIN. These differences were statistically significant for CIN of grades 1 and 2 ($P < 0.025$ to $P < 0.0001$) but not for CIN of grade 3 (Tables 3 and 4).

We tried to confirm the histological data by performing Western immunoblotting on microdissected lesions. The latter were obtained by a laser capture microdissection system from paraffin-embedded cervical tissue sections of HIV-negative and HIV-positive women. Unfortunately, we were unable to detect protein bands corresponding to HGF and c-Met on immunoblots. It is possible that this was because of the known cross-linking effects of formalin on proteins, and/or the amount of proteins obtained from the microdissected lesions was too low to be detected.

Investigations of Normal Tissue Controls and of Lesions Infected with Nononcogenic HPV Underlines the Influence of HPV and HIV. Additional studies performed on a small cohort of condyloma acuminata displaying nononcogenic HPV(s) from HIV-negative and HIV-positive patients, and on two sets of specimens of cervix mucosa exhibiting normal appearance, either from healthy women or corresponding to areas contiguous to oncogenic HPV lesions from women with HIV-negative or -positive status, provided new findings of interest. Three observations emerge from quantitative data (Table 5).

First, the mean percentage of cell layers displaying immunostaining for c-Met did not vary among normal mucosa from healthy women, histologically normal mucosa from tissues adjacent to the CIN containing oncogenic HPV(s), and condyloma acuminata, if these last two types of sample were taken from HIV-negative women. Similarly, the percentage of cell layers displaying immunostaining for HGF was low, a mean of one or two cell layer(s), and similar for normal mucosa from healthy women (Fig. 6a) and condyloma acuminata from HIV-negative women (Table 5). In all of these samples, the immunostaining intensity was generally faint.

Second, if we considered HIV-negative women alone, the estimated percentages of labeled cells were 8% for HGF and 23% for c-Met in condyloma acuminata only displaying HPV6 and 11, and reached 63% for HGF and 61% for c-Met, in cervical lesions of comparable low-grade dysplasia (CIN of grade 1) but infected with oncogenic HPV(s; compare Table 5 with Tables 3 and 4). The strong increase in expression for the two proteins detected in the last lesions was probably entirely because of the sole presence of oncogenic HPV(s).

Third, although we studied only a small population of condyloma acuminata, it was clear that the percentage of cell layers immunostained for HGF and c-Met was dramatically enhanced ($P < 0.02$ to $P < 0.01$) in those lesions originating from HIV-positive women as compared with HIV-negative women. Similarly, c-Met expression was significantly higher in the histologically “normal” mucosa contiguous to lesions in women coinfected with oncogenic HPV and HIV ($P < 0.01$), than in the corresponding tissue from women infected with oncogenic HPV only (Table 5). This fact likely suggests the presence of relatively numerous copies of oncogenic HPV in the normal-appearing mucosa in the coinfected patients. It is consistent with the persistent HPV infection observed in the cervical epithelium of women, especially with HIV infection (7), and the multiple recurrences of HPV infection after CIN treatment proven in HIV-infected population (28). Nevertheless, the increase noted in this “normal” mucosa was smaller than that noted in oncogenic HPV lesions themselves, the percentage of cell layers labeled reaching ~43%, versus 73–84% in oncogenic HPV lesions (compare Table 5 with Table 3). Globally, these observations underlined again the influence of the HIV-positive status.

A last point concerns the pattern of HGF expression in the “normal” mucosa adjacent to lesions containing oncogenic HPV(s), which differed from the pattern of expression of c-Met. Indeed, whatever the HIV status, the percentage of cell layers labeled increased similarly (by a factor of ~10) with respect to the normal value for healthy women (Table 5; Fig. 6, b and c).

In Situ RT-PCR on CIN Lesions Indicates the Presence of c-Met mRNA Transcripts in the Pathological Epithelium. Correct peptide mRNA preservation in tissues is more difficult to obtain than that of viral particles and needs to take particular precautions during the histological process. Nevertheless, we attempted to look for the presence of c-Met and HGF mRNAs in

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**Table 5** c-Met and HGF immunostaining in normal squamous mucosa of the uterine cervix and in condyloma acuminata specimens without oncogenic HPV

Student’s $t$ test was used if SDs were similar in the two groups compared and the Mann-Whitney $U$ test was used if they differed significantly. Values are means ± 1 SEM.

<table>
<thead>
<tr>
<th></th>
<th>No. cell layers</th>
<th>c-Met percentage immunostained layers</th>
<th>HGF percentage immunostained layers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa in healthy women ($n = 10$)</td>
<td>21.3 ± 1.8</td>
<td>30.7 ± 7.3</td>
<td>4.6 ± 2.2</td>
</tr>
<tr>
<td>Condyloma acuminata with nononcogenic HPV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV− ($n = 6$)</td>
<td>26.0 ± 5.0</td>
<td>23.3 ± 12.5</td>
<td>7.9 ± 2.3</td>
</tr>
<tr>
<td>HIV+ ($n = 5$)</td>
<td>33.3 ± 1.0</td>
<td>80.2 ± 11.5*</td>
<td>56.6 ± 11.1*</td>
</tr>
<tr>
<td>Normal mucosa, adjacent to CIN with oncogenic HPV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV− ($n = 10$)</td>
<td>20.8 ± 1.4</td>
<td>16.0 ± 5.2</td>
<td>44.9 ± 12.9</td>
</tr>
<tr>
<td>HIV+ ($n = 13$)</td>
<td>23.4 ± 2.2</td>
<td>42.6 ± 9.4*</td>
<td>40.0 ± 9.7</td>
</tr>
</tbody>
</table>

* $p < 0.02$ versus corresponding HIV-negative women.
* $p < 0.01$ versus corresponding HIV-negative women.
CIN lesions from our fixed archival material using the in situ RT-PCR technique. We succeeded in detecting c-met mRNA transcripts in some lesions, although in relatively low levels. They were expressed predominantly by koilocytes (Fig. 7a).

HGF mRNA transcripts were not detected in serial adjacent sections of the same lesions, mounted on the same slide. Omission of Taq polymerase and primers resulted in the absence of signal in the tissues (Fig. 7b).

**DISCUSSION**

The present study provides quite original findings about the expression and distribution of HGF and c-Met in condyloma acuminata and flat intraepithelial lesions of the cervix, and highlights for the first time the role that viruses such as oncogenic HPVs and HIV may play in the overexpression of the HGF/c-Met complex in cervical disease.

This study confirms that the normal squamous epithelium of the female genital tract expresses HGF protein (8) and shows that normal uterine cervix also expresses c-Met protein, with both of these proteins produced in small amounts. Findings particularly interesting concern the pathological mucosa. Indeed, in HIV-negative women, it is first evident that the levels of HGF and c-Met in squamous cervical lesions infected with nononcogenic HPV(s; i.e., in acuminata condylomas considered as low-grade SIL or CIN of grade 1) did not differ from those in normal mucosa, whereas the sole presence of oncogenic HPVs in CIN of the same grade was statistically associated with the overexpression of these proteins, because no other infection either bacterial or viral in nature was detected in these lesions. Few condyloma acuminata specimens were examined with respect to the number of CIN studied. However, it must be pointed out that it is difficult to obtain condyloma acuminata samples from the cervix because: (a) they were relatively rare in that location by comparison to vulva; and (b) such lesions are typically identified on endoscopic examination and in most cases destroyed by laser. Secondly, our findings indicate that overall, HGF and c-Met levels were significantly correlated with the severity of the intraepithelial lesions infected with oncogenic HPV(s); however, no relationship was observed between the levels of HGF and c-Met in squamous cervical intraepithelial lesions and any of the specific oncogenic HPV types.

HIV infection has been shown to increase the relative risk of anal carcinoma (5, 6). It is known that HIV does not infect squamous cells per se but is present in small amount in Langerhans cells of squamous tissues, such as esophageal (29), anal (6), and cervical mucosae (30). Herein, we found that HIV infection is accompanied by high levels of HGF and c-Met immunostaining in the pathological mucosa of the uterine cervix either infected or not with oncogenic HPV(s). Moreover, the increase with HIV infection appeared to be more pronounced than that with oncogenic HPV(s), as significantly higher number and percentage of HGF and c-Met immunostained cell layers were observed in the coinfected population for CIN1 and CIN2, indicating that there is a considerable increase in the absolute number of positive cells (Tables 3 and 4). The intensity of immunostaining for the two proteins was also stronger in HIV-infected population (Figs. 4 and 5). However, there was no clear relationship between seric immune status, and HGF and c-Met levels, suggesting that the influence of HIV may be mediated by the progressive loss of local immune cell population. Although there was an increase in HGF and c-Met expression during the CIN3 stage in HIV-infected patients as compared with HIV-negative ones, the difference between the two groups was not significant. This could be explained merely by the smaller number of CIN of this grade examined in both groups of patients, resulting in elevated SEM or by a lesser HIV influence on high dysplasia of grade 3 when oncogenic HPV DNA is integrated into host cell DNA.

Although it is tempting to suggest a direct role for HPV and HIV in HGF and c-Met overexpression, our present data show association and not causation. Nevertheless, the influence of
viruses on the expression of several genes has already been reported. For instance, it was found recently that expression of latent membrane protein-1, a protein encoded by EBV, is significantly correlated with c-Met expression in nasopharyngeal carcinoma and that transfection of latent membrane protein-1 expressing plasmid into Madin-Darby canine kidney epithelial cells induces c-Met protein expression in these cells (31). Changes in the pattern of expression of E-cadherin during the progression of SILs of the cervix have been reported (32–35), and experimental data in HPV-transfected squamous cells have suggested that alterations in E-cadherin expression may be directly related to infection with oncogenic HPV (36). As, in a previous study, we observed no difference in the pattern of E-cadherin expression in oncogenic HPV-infected CIN between HIV-positive and HIV-negative women (35), one may suggest that, unlike oncogenic HPV(s), HIV infection and the immune status have no effect on this variable. In contrast to these data, we found in the present study that, in two other groups of patients, the level of HGF and c-Met expression did seem to be affected in relation with HIV infection. Finally, whatever the virus considered, HPV or HIV, our findings show that changes in the pattern of HGF/c-Met expression occurred early in the multistage process of squamous cervix carcinogenesis, as soon as CIN of grade 1 and even the normal-appearing mucosa adjacent to lesions. Of particular interest, COX2 and other genes have recently been found up-regulated in normal mucosa adjacent to cervical dysplasia or cancer but not in normal mucosa from cervical disease-free patients (37, 38). Alteration in gene expression profiles in apparently normal cervical tissue adjacent to tumor may be indicative of early neoplastic or progressive changes that eventually lead to invasive cervical cancer.

We report here the colocalization of HGF and c-Met proteins in the pathological squamous cervical epithelium. In a first hypothesis, the presence of the HGF protein in this epithelium may reflect HGF binding to the target cells via its receptor, particularly if we assume that HGF, produced by mesenchymal cells, acts in a paracrine manner. Alternatively, HGF may be synthesized in the infected epithelium, constituting an autocrine activation loop with c-Met. The detection, using ISH, of HGF mRNAs in some epithelial tissues of the human fetus (10), and in epithelial cells of normal lung and primary non-small-cell lung cancers (11), is consistent with the second hypothesis. Therefore, to check these hypotheses, we performed in situ RT-PCR on cervical tissues. We did observe c-Met mRNA expression, although in low level, in some epithelial lesions; however, we did not detect HGF mRNA in the same lesions. Many conditions are required for mRNA preservation, particularly the use of frozen sections is often mandatory. Fixation parameters such as fixation delay, time, and temperature may account for large expression variability of genes. Because these conditions from our paraffin-embedded tissue blocks were not optimum, we cannot definitively conclude in the absence of HGF gene expression in the cervical epithelial lesions, HGF mRNA being perhaps less stable and therefore more degraded than c-Met mRNA. RT-PCR performed on fresh cervical tissues could theoretically answer the question; however, we did not perform it, because major precautions must be taken when studying fresh virus-infected tissues, particularly if these tissues are obtained from HIV-positive patients. Whatever the origin of HGF proteins in the pathological mucosa of the uterine cervix, their presence in high amounts may lead to c-Met activation and, thus, induce some actions such as the stimulation of cell growth, as it is known that HGF promotes epithelial cell proliferation. This has notably been shown in vitro in human endometrial and ovarian cancer cells (22, 23). The present findings seem to support this hypothesis, because the overexpressed HGF/c-Met complex was always accompanied by an increase in the absolute number of cell layers. This was true for both (a) condyloma acuminata from HIV-infected women compared with HIV-negative ones; and (b) CIN infected with oncogenic HPV(s), even if the mucosal thickness is sometimes reduced, cells being in most cases smaller with poor maturation than in normal mucosa. Nevertheless, a reduction in cell desquamation cannot be totally excluded to explain this increase in cell layers. Because the HGF/c-Met complex also promotes in vitro the migration and morphogenesis of human epithelial cells of the...
genital tract (22–24), particularly of cervical carcinoma cells (25), and is known to be involved in the metastatic process of other tissues (14–16), it is therefore possible that its overexpression also plays a direct role in cervical tumor progression. Informations provided herein may be potentially useful for novel antitumoral therapy inhibiting the c-Met/HGF signaling pathway.

In conclusion, oncogenic HPVs and HIV-seropositivity closely correlate with the overexpression of HGF and c-Met proteins in squamous cervical epithelial lesions. Taking into consideration the various actions of HGF, our data strongly suggest that the overexpressed HGF/c-Met complex contributes to the stimulation of cell proliferation observed in condyloma acuminata and CINs, and, by itself or together with other factors, in the progression of cervical tumor lesions infected with oncogenic HPV(s), notably in HIV-positive women.

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Hepatocyte Growth Factor and c-Met in Cervical Intraepithelial Neoplasia: Overexpression of Proteins Associated with Oncogenic Human Papillomavirus and Human Immunodeficiency Virus

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