Colonization of *in Vitro*-Formed Cervical Human Papillomavirus-Associated (Pre)Neoplastic Lesions with Dendritic Cells

Role of Granulocyte/Macrophage Colony-Stimulating Factor

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The purpose of this study was to investigate the ability of CD1a⁺ Langerhans/dendritic cells (LCs/DCs) to infiltrate human papillomavirus (HPV)-associated (pre)neoplastic lesions of the uterine cervix. Migration of LCs/DCs in the presence of keratinocytes derived from normal cervix and HPV-transformed cell lines was evaluated in Boyden chambers and in organotypic cultures and correlated with granulocyte/macrophage colony-stimulating factor (GM-CSF) production by the cells, as determined by ELISA. Conditioned media of HPV-transformed keratinocytes contained lower amounts of GM-CSF and induced a decreased motile response of LCs/DCs in the Boyden chamber assay compared with those of normal cervical keratinocytes. The migration of LCs/DCs in the presence of conditioned media from normal keratinocytes could be blocked by an anti-GM-CSF antibody, and the migration of LCs/DCs in the presence of conditioned media from HPV-transformed keratinocytes could be increased by supplementing the media with recombinant GM-CSF. GM-CSF was also a potent factor in enhancing the colonization of LCs/DCs into organotypic cultures of HPV-transformed keratinocytes, as the infiltration of LCs/DCs in the *in vitro*-formed (pre)neoplastic epithelium was minimal under basal conditions and dramatically increased after the addition of GM-CSF to the cultures. These results suggest that GM-CSF could play an important role in the recruitment of LCs/DCs into the HPV-transformed (pre)neoplastic cervical epithelium and be useful as a new immunotherapeutic approach for cervical (pre)cancers. (*Am J Pathol* 1999, 154:775–784)

Cervical cancer is the second leading cause of cancer in women worldwide.¹ It is now well established that human papillomavirus (HPV) plays a major role in the develop-

ment of cervical cancer and its precursors (squamous intraepithelial lesions (SILs)).² Additional environmental and/or host factors are probably involved in malignant progression as suggested by the isolation of HPV DNA from normal cervical cell samples,^{3,4} the small number of infected individuals developing cervical cancer, and the relatively long latency period before cancer emergence.⁵

Although the nature of an effective immune response to HPV infection is not well understood, humoral immunity to HPV seems to be a reflection of tumor progression, not a protective immune response.⁶ In contrast, several lines of evidence suggest that cellular immunity is important in controlling HPV-associated SILs.⁷ Indeed, several studies have described a localized immune dysfunction accompanying cervical HPV infections.^{8,9} Quantitative and qualitative alterations of CD4⁺ T lymphocytes have been reported.^{5,10,11} Moreover, the number, distribution, and morphology of Langerhans cells (LCs) have been shown to be altered in HPV-infected (pre)neoplastic cervical epithelium.¹²

LCs belong to the lineage of dendritic cells (DCs) that constitute a network of sentinels considered to be the most important professional antigen-presenting cells in the immune system. Currently, DCs can be obtained from monocytic precursors (CD14⁺).¹³ They can be induced to proliferate and to differentiate into DCs (CD1a⁺/CD14⁺) in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. LCs/DCs are known to play key roles in the initiation and regulation of cutaneous, mucosal, and systemic immunity.¹⁴ LCs infiltrate different tumors, including cervical cancer, and have been shown to be associated with a

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better prognosis.¹⁵⁻¹⁷ This observation, associated with the decreased density of LCs in most cervical SILs, suggests that a quantitative and/or qualitative disturbance of LCs could interfere with the immune surveillance of HPV-associated cervical (pre)neoplastic lesions.

The epithelial microenvironment is known to play an important role in determining the density and/or function of LCs.^{18,19} However, little is known about the factors controlling the number, distribution, and functional activity of LCs in HPV-associated (pre)neoplastic lesions of the cervix. Several hypotheses have been proposed to explain the local deficiency of LCs observed in most SILs. Some authors have suggested a cytopathic effect of the virus on LCs.²⁰ Others have hypothesized that the altered expression of E-cadherin, as observed on HPV-infected keratinocytes, contributes to the reduction in number and function of LCs.^{21,22} Another possibility is that the immunosuppressive cytokine IL-10, which has been shown to be highly expressed in SILs,²³ could play a role in the functional inhibition of LCs.²⁴ An additional interesting hypothesis is based on the findings that keratinocytes can produce immunoregulatory cytokines that are likely to have a stimulatory effect on LCs, such as tumor necrosis factor (TNF)- α or GM-CSF.²⁵ Transformation of keratinocytes with HPV has been shown to alter this function,²⁶ causing a reduction of cytokine production that may influence the distribution and differentiation of LCs.

GM-CSF is an important factor not only for the maturation and differentiation of LCs/DCs but also for their motility.^{27,28} GM-CSF produced by keratinocytes acts as a selective chemoattractive stimulus for the continual migration of LCs/DCs into the epithelium. Exposure of LCs/DCs to GM-CSF *in vitro* prolongs their survival and increases their capacity to present antigens to lymphocytes.²⁹ *In vivo*, a correlation has been observed between the amount of GM-CSF produced by some carcinomas and the distribution/differentiation of tumor-associated DCs.^{30,31}

The current study was designed to evaluate the capacity of GM-CSF to influence the migratory capacity of *in vitro* generated DCs in Boyden chambers and in organotypic cultures of HPV-transformed keratinocytes. The organotypic culture of keratinocytes has been used previously to examine the effects of therapeutic agents on a variety of malignant keratinocytes³²⁻³⁵ or as a model for immuno-pharmaco-toxicological studies.³⁶ In this system, keratinocytes are grown at the air-liquid interface on top of a dermal equivalent support. The normal keratinocytes stratify and exhibit a typical pattern of differentiated squamous epithelium, whereas HPV-transformed and established squamous carcinoma cell lines exhibit morphologies similar to those of high-grade lesions seen *in vivo*.^{37,38} So, the organotypic culture system of HPV-transformed keratinocytes might be particularly suited to examine the effects of new therapeutic strategies and particularly to investigate the interactions between cervical (pre)neoplastic lesions and various types of immuno-competent cells, among them LCs/DCs. Because three-dimensional cultures are obtained under these conditions, the ability of LCs/DCs to infiltrate HPV-transformed (pre)neoplastic epithelium may be assessed by a

quantitative image analysis of immunohistochemical markers (CD1a).

Materials and Methods

Culture of Normal Cervical Keratinocytes

Human exocervical epithelial cells were obtained from hysterectomy specimens of healthy women. Cell cultures were established and maintained following a previously reported method.³⁹ Briefly, tissue fragments were incubated with 0.25% trypsin (GIBCO BRL, Gaithersburg, MD) for 24 hours at 4°C. The epithelial cells were detached and cultured with irradiated 3T3 mouse fibroblasts as a feeder. The growth medium was a 1/3 mixture of HAM F12 (GIBCO BRL)/Dulbecco's modified Eagle's medium (GIBCO BRL), supplemented with 0.5 μ g/ml hydrocortisone (Sigma Chemical Co., St Louis, MO), 10 ng/ml epidermal growth factor (Sigma), 10% fetal calf serum (Life Sciences International, Zellik, Belgium), 2 mmol/L L-glutamine (GIBCO BRL), 10 mmol/L Hepes (GIBCO BRL), 1 μ g/ml fungizone (GIBCO BRL), 1 mmol/L sodium pyruvate (GIBCO BRL), 3000 U/ml penicillin-streptomycin (GIBCO BRL), 10^{-10} mol/L cholera toxin (Sigma), 5 μ g/ml insulin (Sigma), 20 μ g/ml adenine (Sigma), 5 μ g/ml human transferrin (Sigma), and 15×10^{-4} mg/ml 3,3',5-triiodo-L-thyronine (Sigma). Subconfluent cultures were dispersed with 0.0025% trypsin and 0.02% EDTA. These primary cultures of keratinocytes were used for the organotypic cultures. This study protocol was approved by the Ethics Committee of the University Hospital of Liège.

HPV-Transformed Keratinocyte Cell Lines

SiHa, CasKi, and C4-II are tumorigenic cervical carcinoma-derived keratinocyte cell lines.⁴⁰⁻⁴² The SiHa cell line contains one copy of integrated HPV-16 DNA. Three different passages of this cell line were used (SiHa I, SiHa II, and SiHa III), which differed in their production of GM-CSF, as measured by ELISA.

The CasKi cell line contains approximately 600 copies of integrated HPV-16 DNA, whereas the C4-II cell line contains HPV-18 DNA sequences.

The CK2 cell line was established by transfection of human cervical keratinocytes with HPV-33 DNA and is not tumorigenic in nude mice.³⁹

All of these HPV-transformed keratinocyte cell lines were grown and maintained in a mixture of HAM F12 and DMEM supplemented with the same additives as those used for normal keratinocyte cultures.

Dendritic Cell Cultures

DCs were generated by culturing adherent fractions of human peripheral blood mononuclear cells as previously described.^{13,43} Briefly, mononuclear cells were isolated from leukocyte-enriched buffy coats by centrifugation on Ficoll-Hypaque. The plastic adherent fraction (10×10^6 cells/well, 18 hours at 37°C) was cultured with 800 U/ml

human recombinant GM-CSF (kindly provided by Schering-Plough, Brussels, Belgium) and 100 U/ml IL-4 (Genzyme Diagnostics, Cambridge, MA) in 3 ml of RPMI containing 10% FCS/50 μ mol/L mercaptoethanol. Cultures were fed every 3 days with fresh medium containing cytokines and harvested at day 7. Cells of each culture were analyzed by flow cytometry and displayed the previously described surface phenotype of DCs (HLA-DR⁺, MHC class I⁺, CD1a⁺, CD4⁺, CD54⁺, CD80⁺, CD86⁺, CD3⁻, and CD14⁻).^{13,43}

Organotypic Cultures

Organotypic cultures of normal cervical keratinocytes and HPV-transformed keratinocytes were prepared by procedures slightly modified from those described previously.^{32,44} For the preparation of dermal equivalents, a collagen matrix solution was made with 32 mg of collagen (Cellagen solution AC-5, type I, ICN, Biomedical, Asse-Relegen, Belgium) mixed on ice with 1.6 ml of 0.1% acetic acid, 1 ml of chilled 10-fold concentrated Hanks' buffer supplemented with phenol red and 1 N NaOH to give a pH of 7.2. One milliliter of FCS containing 4×10^5 normal human fibroblasts was then added. One milliliter of the collagen/fibroblast solution was poured into 24-well plates (Nuncclon Δ Multidishes, Nunc, Roskilde, Denmark) and allowed to solidify at 37°C for 1 hour. The final concentrations of collagen and fibroblasts were 3.2 mg/ml and 4×10^4 cells/ml, respectively. After gel equilibration with 1 ml of growth medium overnight at 37°C, 25×10^4 to 1×10^6 keratinocytes (normal or HPV-transformed) resuspended in 100 μ l of growth medium were seeded on top of the gels and maintained submerged for 24 to 96 hours. The collagen rafts were raised in a 25-mm tissue culture insert (8- μ m pore size; Nunc) and placed onto stainless-steel grids at the interface between air and liquid culture medium. Epithelial cells were then allowed to stratify over 2 weeks. After stratification of keratinocytes, DCs were seeded on top of the *in vitro*-formed epithelium at a concentration of 2×10^5 cells/50 μ l of growth medium, and organotypic culture medium was supplemented or not with 800 U/ml GM-CSF. After 24 hours at 37°C, the collagen rafts were harvested. The cultures were then embedded in OCT compound (Tissue Tek, Sakura, The Netherlands) at -70°C and sectioned with a cryostat microtome for the immunohistochemical analysis.

Immunohistochemistry

The density of DCs migrating into the epithelial layer was assessed by the avidin-biotin-peroxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with an anti-CD1a monoclonal antibody (clone NA1/34 from Dako, Glostrup, Denmark). Nine-micron frozen sections were fixed in cold acetone for 3 minutes, and endogenous peroxidases were blocked with 0.1% H₂O₂ for 30 minutes. Sections were then incubated sequentially with anti-CD1a antibody (at a 1/40 dilution in PBS containing 2% bovine serum albumin (BSA) and

0.01 mol/L NaN₃) or with an isotype-matched control antibody for 1 hour, with a biotinylated mouse anti-Ig antibody for 30 minutes, and with streptavidin/horseradish peroxidase/avidin/biotin complex for another 30 minutes. Positive cells were visualized by a 3,3'-diaminobenzidine substrate (DAB). The sections were counterstained with hematoxylin.

Direct double-immunofluorescence staining was performed to evaluate the relative proportion of macrophages (CD14⁺) and DCs (CD1a⁺) in the epithelial layer. Phycoerythrin-conjugated anti-CD14 (Leu-M3, IgG2b; Becton Dickinson, Erembodegem, Belgium) and fluorescein-isothiocyanate-conjugated anti-CD1a (OKT6; Ortho, Raritan, NJ) were used at a 1/10 dilution.

Assessment of CD1a⁺ Cell Infiltration in Organotypic Cultures

The DC infiltration in organotypic cultures was evaluated by measuring the surface of immunostained cells with a computerized system of image analysis (CAS, Becton Dickinson) following a method previously described.³² The entire surface of each culture section was analyzed. To determine the number of CD1a⁺ cells within a given culture, three sections stained with anti-CD1a were counted. The positive surface was expressed as the percentage of the total surface analyzed. Statistical analysis was performed by using the Student *t*-test (Instat Mac 2.01 software; Graph-Pad Software, San Diego, CA) for each separate cell line or normal keratinocyte culture.

Chemotaxis Assay Using Keratinocyte-Derived Cell-Conditioned Media

Chemotactic migration of the DCs was tested as previously described.⁴⁵ Briefly, after phenotypic characterization, DCs were resuspended in serum-free growth medium containing 0.1% BSA. Polyvinylpyrrolidone-free polycarbonate membrane 8- μ m pore filters (Poretics Corp., Livermore, CA) were coated by incubation with 100 μ g/ml gelatin in 0.1% acetic acid solution and placed in a chemotactic Boyden microchamber (Neuroprobe, Cabin John, MD). The lower compartment was filled with 27 to 28 μ l of keratinocyte-derived cell-conditioned medium, obtained from subconfluent keratinocyte cultures, containing 0.1% BSA. Nonconditioned medium was used as a control for random migration. Each condition was repeated six times, and 55 μ l of DC suspension (2×10^6 cells/ml) was added to the upper compartment of the chamber. The chambers were incubated for 5 hours at 37°C in a 5% CO₂/95% air atmosphere. The filters were removed, fixed, and stained using the Diff-Quick stain set (Baxter Diagnostics, Düringen, Switzerland), and the upper side of the filter was scraped to remove residual nonmigrating cells. Two random fields were counted per well using an eyepiece with a calibrated grid to evaluate the number of fully migrated cells.

Figure 1. GM-CSF secretion by cultures of cervical epithelial cells. Cultures of exocervical cells (four different donors, KN1 to KN4) and HPV-transformed cell lines were tested. Values represent the mean of three independent experiments $\pm$ SD.

ELISA

The amount of GM-CSF in keratinocyte cultures was measured by ELISA (Medgenix, Fleurus, Belgium) using supernatants from normal keratinocytes and HPV-transformed cell lines collected from subconfluent cultures after 48 hours.

Results

Decreased Production of GM-CSF by Cervical Carcinoma-Derived Cell Lines Compared with Normal Exocervical Keratinocytes

GM-CSF was quantified by ELISA using supernatants collected from subconfluent cultures after 48 hours. Normal keratinocyte cultures, derived from four different individuals, were tested at the second passage to avoid any possible contamination with feeder cells. Figure 1 shows that the spontaneous production of GM-CSF by HPV-transformed cell lines was uniformly low. In contrast, keratinocytes from the normal exocervix released substantial amounts of GM-CSF, up to 95-fold higher than cervical carcinoma-derived cell lines. Similar results were obtained when using cultures maintained in medium without FCS, although the secreted quantities of GM-CSF were systematically lower in the absence of serum (data not shown).

Chemotaxis of DCs in the Presence of Conditioned Media of HPV-Transformed Cell Lines

To determine whether conditioned media of HPV-transformed keratinocytes could influence the migration of DCs, we conducted a migration assay using a Boyden microchemotaxis chamber. DCs generated for this study were judged to be 90% pure based on several criteria, including morphology, forward-scatter and side-scatter values observed by FACS analysis, and surface phenotype (data not shown).

As shown in Figure 2, conditioned media from normal keratinocytes significantly enhanced the chemotaxis of

Figure 2. Chemotactic activity of conditioned media of keratinocytes on DCs. NC, nonconditioned medium; KN1 and KN2, conditioned media of normal keratinocytes from two different donors; F. Hum, conditioned medium of human fibroblasts (positive control). Other conditioned media came from HPV-transformed cell lines. Results are the mean $\pm$ SD of four experiments.

DCs, compared with the nonconditioned medium used to assess the chemokinesis. The chemotactic activity of conditioned media from normal keratinocytes was similar to the level of chemotaxis induced by the conditioned medium of human fibroblasts, known to produce chemoattractants for DCs.⁴⁶

In contrast, the motility of DCs in the presence of conditioned media from HPV-infected keratinocytes was significantly diminished compared with that in the presence of conditioned media from normal keratinocytes. The motility of DCs in the presence of conditioned media from HPV-transformed keratinocytes was similar compared with the migration in nonconditioned media. The diminished production of GM-CSF as detected by ELISA in the conditioned media of HPV-transformed keratinocytes (Figure 1) correlated with the reduced chemotaxis of DCs, except for two cell lines (C4 and CK2) that secreted lower quantities of GM-CSF than SiHa I but were associated with a higher level of DC penetration.

The staining of polycarbonate membranes from chemotaxis chambers showed cells with DC morphology (cytoplasmic veils) crossing the membrane by protruding long and thick cytoplasmic processes through the filter pores. In contrast, the morphology of DCs incubated with conditioned media of HPV⁺ cell lines displayed few cytoplasmic processes (data not shown).

Effect of Anti-GM-CSF and Recombinant GM-CSF on the Chemotactic Activity of Conditioned Media

To ascertain whether the chemotactic activity of the conditioned media was related to their content of GM-CSF, conditioned media were pretreated with an anti-GM-CSF antibody before assaying their chemotactic activity on DCs in Boyden chambers (Figure 3A). We observed that the anti-GM-CSF effectively blocked the motile response of DCs incubated with conditioned media from normal keratinocytes. There was approximately a 50% decrease in the migration of DCs in the presence of conditioned media pretreated with anti-GM-CSF antibody. The values obtained when using the antibody were similar to that obtained with nonconditioned medium. The anti-GM-CSF antibody also affected the motile response of DCs in the

Figure 3. A: Effect of anti-GM-CSF antibody on the chemotactic activity of conditioned media of keratinocytes. B: Effect of exogenous GM-CSF addition on the chemotactic activity of conditioned media of SiHa cell line. NC, nonconditioned medium; F. Hum, conditioned medium of human fibroblasts (positive control); KN1, KN3, and KN4, conditioned media of normal keratinocytes from three different donors. Asterisks indicate statistically significant differences: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$.

presence of conditioned media of the HPV-transformed cell lines C4, CK2, and Caski but had no effect on the chemotaxis of DCs incubated with conditioned media of the SiHa I, which is characterized by a very low basal production of GM-CSF.

We next investigated whether the addition of GM-CSF to the conditioned media of HPV⁺ cell lines could restore the migration of DCs (Figure 3B). Recombinant human GM-CSF was added to conditioned media to obtain a quantity of GM-CSF similar to that detected in the conditioned media of normal keratinocytes. The chemotactic activity induced by the conditioned media of HPV⁺ cell lines supplemented with GM-CSF was significantly increased and reached the chemotaxis activity obtained with the conditioned media of normal keratinocytes.

GM-CSF Influences the Infiltration of DCs into Organotypic Cultures of HPV-Transformed Keratinocytes.

We investigated whether the addition of exogenous GM-CSF could modulate the ability of DCs to infiltrate an *in vitro*-formed (pre)neoplastic epithelium, reminiscent of a cervical high-grade SIL observed *in vivo*.

After 1 to 2 weeks of culture, the SiHa cell line (and all of the cell lines used in this study), grown on a collagen gel at the air/liquid interface, produced an epithelial layer of more than 10 cells in thickness that closely resembled a high-grade cervical lesion (Figure 4, C and D). In contrast, normal keratinocytes formed a three-dimensional structure that closely resembled a normal exocervical epithelium (Figure 4, A and B). DCs were layered on the top of these cultures with or without the addition GM-CSF. The effect of GM-CSF on DC infiltration was assessed by examining immunohistological sections of organotypic cultures at 24 hours after GM-CSF addition. The ability of GM-CSF to influence the infiltration of DCs was determined by the evaluation of CD1a⁺ cells. Figure 4, E–H, illustrates representative experiments showing the density of CD1a-labeled DCs in normal and HPV-transformed organotypic cultures incubated with or without exogenous GM-CSF.

In organotypic cultures of normal keratinocytes, DCs migrated in a similar manner in the presence or in the absence of GM-CSF (Figure 4, E and F). In contrast, DCs layered onto organotypic cultures of SiHa poorly infiltrated the epithelial layer in the absence of GM-CSF (Figure 4G), whereas addition of GM-CSF caused a significant increase in the density of DCs observed in the epithelial layer (Figure 4H).

Quantitative analysis of DC infiltration was performed on organotypic cultures derived from six HPV-transformed cell lines, which expressed different quantities of endogenous GM-CSF (Figure 1) and on three organotypic cultures derived from normal keratinocytes as controls. The density of DCs in the *in vitro*-formed epithelium was quantified by evaluating the surface labeled with the anti-CD1a antibody throughout the full thickness of organotypic cultures. Under basal conditions (Figure 5A), the level of DC infiltration in cultures of normal keratinocytes was higher than in HPV⁺ cell lines cultures. Except for the Caski cell line, a positive correlation was observed for the number of CD1a⁺ cells present in the epithelial layer and the amount of GM-CSF produced by the HPV-transformed cell lines ($r^2 = 0.85$; $P = 0.0004$; data not shown).

When the medium of organotypic cultures of HPV⁺ cell lines was supplemented with GM-CSF (Figure 5B), the infiltration of DCs improved and reached an infiltration level equivalent to that obtained with normal keratinocytes. The infiltration of DCs in the presence of GM-CSF was significantly higher for most cell lines compared with that observed in the absence of GM-CSF. In contrast, Caski, which secreted higher levels of GM-CSF, was surprisingly associated with a lower infiltration of DCs. The addition of exogenous GM-CSF was not as effective with Caski, as with the other cell lines, in improving DC penetration. In contrast, the density of DCs observed in organotypic cultures of normal keratinocytes was slightly lower for some cultures in the presence of exogenous GM-CSF.

Similar observations were made using different numbers of DCs and/or prolonging the incubation time (up to 1 week; data not shown).

Figure 4. SiHa cell and normal keratinocyte organotypic cultures as models of high-grade cervical lesions and normal epithelium, respectively (A to D, H&E). A: Biopsy specimen of normal cervical epithelium. B: Section of an organotypic culture of normal cervical keratinocytes. C: Biopsy specimen of high-grade cervical SIL. D: Section of an organotypic culture of SiHa cells. E to H: Correlation between the penetration of CD1a⁺ cells into organotypic cultures and the addition of exogenous GM-CSF, shown by CD1a⁺ immunolabeled sections of organotypic culture of normal keratinocytes (E and F) and SiHa cells (G and H) in the absence (E and G) or in the presence (F and H) of GM-CSF.

Figure 5. Quantitative evaluation of DC infiltration into organotypic cultures of normal and HPV-transformed keratinocytes. The penetration of DCs under basal conditions (A) and in the absence or in the presence of recombinant GM-CSF (B) is followed by immunolabeling with anti-CD1a. Results are expressed as percentages of surface labeled with anti-CD1a compared with unlabeled surface of the epithelial sheet $\pm$ SD ($n = 4$ for each HPV-transformed cell line and normal keratinocyte culture). Asterisks indicate statistically significant differences: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$).

Phenotype of DCs Infiltrating Organotypic Cultures

To determine the potential influence of HPV-transformed keratinocytes on DC differentiation, we performed a double-immunofluorescent staining (CD1a/CD14) of organotypic cultures. Table 1 illustrates results obtained with organotypic cultures of SiHa cells and normal keratinocytes. The phenotype of DCs was established before their addition onto organotypic cultures and after 24 and 48 hours of infiltration in the presence or in the absence of GM-CSF.

DCs infiltrating organotypic cultures of SiHa in the presence of GM-CSF were found to be more differentiated, as a higher proportion of CD1a and a reduction of double-positive CD1a/CD14 cells were observed after 24

and 48 hours of infiltration. However, in the absence of exogenous GM-CSF, some DCs seemed to revert to monocytes, as we observed a decreased percentage of CD1a⁺ cells and an increased proportion of CD14⁺ (2% to 10% reverted to CD14⁺) and CD1a⁺CD14⁺ cells (1% to 5% became double positive).

In contrast, the phenotype of DCs layered onto organotypic cultures of normal keratinocytes was similar in the presence or in the absence of GM-CSF. Compared with their phenotype before addition to the culture, we observed an increase in the percentage of CD1a cells and a decrease in the percentage of double-positive cells (CD1a/CD14), whereas the percentage of CD14⁺ cells was unchanged.

Discussion

Previous studies have shown a diminished density of Langerhans cells (LCs) in the transformation zone of the cervix^{5,47,48} and in most cervical SILs^{49,50} compared with the normal squamous mucosa. Qualitative alterations of LCs have also been reported in SILs.⁵¹ On the other hand, we have shown that the ability to generate dendritic cells (DCs) *in vitro* from blood precursors is essentially the same in healthy women and women with SIL.⁴³ These results suggest that local factors in the cervix play an important role in the development of LC alterations during the HPV-associated cervical carcinogenesis. Because of the central role of LCs/DCs in the generation of an effective cellular immune response, a defect in these cells may contribute to the persistence of HPV and indirectly to the development of cervical cancer. Therefore, we postulated that HPV-transformed keratinocytes could directly influence the number and/or function of LCs by their decreased production of factors necessary for the migration and maturation of DCs.

We demonstrate, by ELISA, that the spontaneous production of GM-CSF by HPV⁺ cell lines is uniformly low. These results are in agreement with a previous study that reported a reduction in the amount of GM-CSF secreted *in vitro* by HPV-infected keratinocytes compared with normal cervical epithelial cells.²⁶

Using a Boyden chamber assay, we show that the motile response of DCs is diminished in the presence of conditioned media from HPV⁺ cell lines compared with that obtained with normal keratinocytes. The supplementation of conditioned media of HPV⁺ cell lines with exog-

Table 1. Phenotype of DCs before and after Infiltration into Organotypic Cultures of SiHa Cell Line and Normal Keratinocytes Supplemented or Not with GM-CSF

Organotypic cultures	Phenotype of DCs	Before	After 24 hours of infiltration		After 48 hours of infiltration	
			-GM-CSF	+GM-CSF	-GM-CSF	+GM-CSF
Normal keratinocytes	CD1a ⁺ /CD14 ⁻	89.1 $\pm$ 0.4	97.1 $\pm$ 1.5	98.7 $\pm$ 6	98.3 $\pm$ 9.4	96.8 $\pm$ 1.5
	CD14 ⁺ /CD1a ⁻	1.2 $\pm$ 0.7	2.7 $\pm$ 1.4	1.2 $\pm$ 3	0.9 $\pm$ 1	2.3 $\pm$ 0.5
	CD1a ⁺ /CD14 ⁺	8.5 $\pm$ 1	0	0	1.5 $\pm$ 0.5	0.6 $\pm$ 0.9
SiHa	CD1a ⁺ /CD14 ⁻	90.4 $\pm$ 0.5	84.5 $\pm$ 6	97.3 $\pm$ 1	85.5 $\pm$ 2	93 $\pm$ 4
	CD14 ⁺ /CD1a ⁻	2.4 $\pm$ 0.7	13.7 $\pm$ 6	2 $\pm$ 0.8	9.2 $\pm$ 3	7.3 $\pm$ 5
	CD1a ⁺ /CD14 ⁺	11.4 $\pm$ 0.3	1.3 $\pm$ 6	0.4 $\pm$ 0.5	5.2 $\pm$ 1	0

Results are expressed as percentages of labeled cells.

enous GM-CSF restored the motility of DCs, whereas the addition of anti-GM-CSF antibody to conditioned media of normal keratinocytes and HPV⁺ cell lines (with the exception of the SiHa cell line) reduced significantly the migration of DCs.

An important finding of this study is that GM-CSF also influenced the infiltration of DCs into an *in vitro*-formed (pre)neoplastic epithelium that resembles high-grade cervical lesions observed *in vivo*. Under basal conditions, the level of DC infiltration in organotypic cultures of normal keratinocytes was higher than in HPV⁺ cell line cultures. We also demonstrated that the addition of exogenous GM-CSF to organotypic cultures of HPV⁺ keratinocyte cell lines significantly increased the infiltration of *in vitro*-generated DCs. In contrast, the addition of GM-CSF into the medium of organotypic cultures of normal keratinocytes had a slight but significant inhibitory effect on DC penetration. This observation may be related to an inhibitory effect of excessive amounts of GM-CSF.⁵²

The precise mechanism of action by which GM-CSF increases the infiltration of DCs in HPV⁺ epithelium remains unclear and requires additional studies. However, two hypotheses may be proposed to explain our results. First, GM-CSF might act as a protective factor counteracting the possible destruction of DCs, as this cytokine has been shown to prolong survival of LCs/DCs *in vitro*.^{28,53} In favor of this hypothesis, we observed modifications in the morphology of DCs incubated in conditioned media of HPV⁺ cell lines compared with conditioned media of normal keratinocytes or conditioned media supplemented with GM-CSF. Second, GM-CSF might also have a chemotactic action on DCs. *In vitro*, GM-CSF has been shown to be capable of enhancing the differentiation, survival, and chemotaxis of DCs^{54,55} and to increase their antigen-presenting capacities.^{56,57} In agreement with these studies, the phenotypic analysis of DCs infiltrating organotypic cultures of SiHa showed an inverse modulation of CD1a and CD14 expression in the absence of exogenous GM-CSF. CD1a expression was found to be low, whereas CD14 was high in cultures not supplemented with exogenous GM-CSF. The up-regulation of CD1a on DCs in the presence of GM-CSF has been previously reported.⁵⁸ In contrast, organotypic cultures of normal keratinocytes were infiltrated by differentiated DCs that stably expressed CD1a probably because of the constitutively high levels of GM-CSF produced by normal keratinocytes. *In vivo*, GM-CSF production in lung carcinoma has been positively correlated with the distribution and differentiation of LCs/DCs.⁵⁹ Additional support for the GM-CSF-induced recruitment of LCs comes from studies showing that the intradermal injection of GM-CSF in patients with leprosy results in a selective accumulation of CD1a⁺ cell in the dermis associated with the migration of these cells from the dermis into the epidermis.^{27,60} Gene therapy studies have also shown that transfection of GM-CSF into tumor cells influences DC recruitment at the tumor site and DC functions in nearby tissues.³¹

Although there is evidence that GM-CSF directly stimulates the migration of LCs/DCs by the induction of a

selective chemoattractive stimulus,^{27,61} we cannot exclude, in our study, the role of other epithelial cytokines, especially for the HPV⁺ cell lines because there was not a strict correlation between GM-CSF production and the migration of DCs. The reason that DC migration, in some cases, was high in the presence of low amounts of GM-CSF remains unknown. As DCs express an array of chemokine receptors⁶² and CC-chemokines induce chemotaxis of human DCs *in vitro*,⁶³ it is possible that monocyte chemoattractant protein-1 or macrophage inflammatory protein-3 α produced by keratinocytes^{64–66} may contribute to the observed results. Moreover, previous studies have demonstrated that cytokines produced by keratinocytes, such as TNF- α , IL-6, IL-8, and IL-1 β , provide signals for migration of LCs to the draining lymph nodes.^{67–69} We are currently investigating the effect of these cytokines or chemokines on the motile response of LCs in the presence of HPV-transformed keratinocytes. Other factors produced *in vivo* by infiltrating leukocytes might also influence the GM-CSF production by epithelial cells and/or act directly on DCs (IL-2⁷⁰ and C5a⁷¹). IL-2 is a cytokine known to attract DCs, to favor their maturation into LCs, and to promote their functional activation. Interestingly, we have previously observed a diminished production of IL-2 in the stroma underlying cervical SILs.⁵ This observation reinforces the hypothesis that LC number may be reduced in SIL due to the lack of chemoattractant factors.

In the organotypic culture model, cell-cell interaction mechanisms could also influence the infiltration of DCs. It has been demonstrated that LCs/DCs adhere to keratinocytes in the epidermis through expression of E-cadherin.²¹ The fact that the expression of this molecule by DCs is down-regulated during their functional maturation *in vitro* suggests that loss of E-cadherin may be an important event in the release of LCs/DCs from the epithelium.⁷² Interestingly, the cell surface expression of E-cadherin was found to be significantly reduced in SILs and most HPV⁺ cell lines.²² Other cell surface molecules, such as integrins or CD44, might also be involved in the influx of DCs to the epithelium.^{73,74}

In summary, we have shown that conditioned media of HPV⁺ cell lines induce a decreased motile response of DCs in a Boyden chamber assay compared with conditioned media from normal keratinocytes. Generally, the migration of DCs was found to be correlated with GM-CSF production by the cultures, to be inhibited by anti-GM-CSF antibody, and to be restored by the addition of recombinant GM-CSF. This effect of recombinant GM-CSF on DC migration was also observed using organotypic cultures of HPV-transformed keratinocytes, which are reminiscent of high-grade SILs observed *in vivo*. These findings suggest that a treatment based on GM-CSF may restore some immunological functions that have been shown to be altered during the progression of cervical SILs. This study also highlights the potential interest of the organotypic culture of HPV-transformed keratinocytes for the design of new immunotherapeutic strategies.

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