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Inverse modulation of intraepithelial Langerhans' cells and stromal macrophage/dendrocyte populations in human papillomavirus-associated squamous intraepithelial lesions of the cervix

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Abstract Ninety-four cervical biopsies from normal tissue to high-grade squamous intraepithelial lesion (SILs) were examined for the presence of intraepithelial Langerhans' cells and subpopulations of stromal macrophages/dendrocytes by immunohistochemistry using anti-S100, -L1, -CD68 and -factor XIIIa antibodies. Human papillomavirus (HPV) detection was performed in all cases by using first a mixture of DNA probes for 14 HPV types commonly found in anogenital biopsies at low stringency conditions (T_m –40°C) and by reanalyzing the tissues at high stringency (T_m –10°C) with HPV 6/11, 16/18 and 31/33/35 biotinylated probe cocktails and individual digoxigenin-labelled probes. SILs and metaplastic tissues were significantly associated with a depletion of S100-positive intraepithelial Langerhans' cells when compared with normal epithelium. In contrast, there was a significant increase in L1-positive stromal macrophages in SIL biopsies compared with normal or metaplastic cervix. A significantly higher density of CD68-positive macrophages was also observed in high-grade SILs compared with normal or metaplastic biopsies and with low-grade SILs. The density of factor XIIIa-positive dendrocytes was found to be higher in SILs compared with metaplastic tissues and in high-grade SILs when compared with normal cervical biopsies. No specific relationship was found between the densities of these cells and the HPV type detected in SILs separated into low grade and high grade. The significance of this inverse modulation of intraepithelial Langerhans' cells and stromal macrophages/dendrocytes in normal and SIL biopsies is discussed in relation to HPV infection and malignant transformation.

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Introduction

Although epidemiological and experimental data support a role for human papillomavirus (HPV) in the development of genital tract neoplasia [43, 78, 83], it is generally considered that HPV is not the sole causative agent and that a number of factors intrinsic to the host may contribute to tumorigenesis. In this context, the general or local immune state might be expected to have a key role in host defence against HPV infection and the possible development of cervical carcinoma. Several studies have shown that the cellular-mediated immunity plays a major role in preventing or limiting the HPV-associated disease [3, 40, 46]. Because cervical neoplasia usually occurs in women with no clinical evidence of systemic immunosuppression, it has been suggested that the local immune reponse within the cervix could be a critical factor in determining the clinical outcome of HPV infection.

Surveillance and defence against viral infections and tumour cells are mediated via both specific and non-specific host immune responses. Among the non-specific effector cells within the cervix, different cell populations of the monocyte/macrophage family, including dendritic cells, may be involved in the cellular reaction to HPV-associated lesions. Potential protective effects might be related to their wide range of functions, including direct cytotoxicity to virus-infected or (pre)neoplastic cells, non-specific phagocytosis, antigen presentation to lymphoid cells or cytokine production [31]. In contrast, macrophages, although they can also present antigens to immunocompetent lymphocytes, have also been shown to promote tumour growth in some situations [34, 41, 64].

The purpose of the present study was to investigate, in the same formalin-fixed paraffin-embedded cervical biopsies, different cell populations of the macrophage/dendrocyte family and to correlate the density of

Table 1 Immunohistochemical panel

Antigen	Antibody	Source	Dilution	Digestion
S100	Polyclonal	Dako[a]	1/100	Pronase E
L1	Monoclonal (Mac387)	Dako	1/300	Trypsin
CD68	Monoclonal (KP1)	Dako	1/100	Pronase E
Factor XIIIa	Polyclonal	Behring[b]	1/350	Pronase E

[a] Dako, Glostrup, Denmark
[b] Behringwerke, Marburg, Germany

these cells with the histological diagnosis of cervical biopsies and HPV type. We used a panel of antibodies useful in the identification of Langerhans' cells (anti-S100), mobile connective tissue histiocytes (anti-L1, CD68) and resident dendrocytes (anti-factor XIII). The antiserum for S100 protein has been shown to be a reliable immunohistochemical marker for Langerhans' cells [72, 74]. The L1 antigen is a myelomonocytic antigen detected by the monoclonal antibody Mac 387 [11, 23]. This antibody reacts with a subset of reactive tissue macrophages and has also been shown to detect the calmogranulin expressed by keratinocytes of differentiated mucosal squamous epithelia [12, 21] and inflamed epidermis [42]. CD68 is a lysosomal antigen expressed in activated cells of the human monocyte-macrophage lineage [60, 69] and is currently used as a pan-macrophage marker [65, 79]. Factor XIIIa is found within a variety of resident dendrocytes in connective tissues as well as in platelets and megakaryocytes [16]. Some classes of monocytes and macrophages may also contain factor XIIIa [14, 38]. The characteristics of factor XIIIa-positive cells have been studied most extensively in human skin, in which there is a population of dermal dendritic cells that bind antibodies against factor XIIIa [6, 15, 36, 37, 62, 63]. They have been shown to display phagocytic functions [5, 16], to act as antigen-presenting cells [15, 37] and to be involved in regulatory mechanisms of deposition of extracellular macromolecules in the dermis [61]. The presence of factor XIIIa-positive dendrocytes has also been reported in close apposition to skin carcinoma and malignant melanoma [5, 6, 29].

Materials and methods

A total of 49 formalin-fixed, paraffin-embedded cervical biopsy specimens of HPV-associated squamous intraepithelial lesions (SILs) were retrieved for our study. These specimens were selected on the basis of both an original diagnostic report indicating that the squamous epithelium had some evidence of HPV infection (condyloma or cervical intraepithelial neoplasia) and the presence of HPV DNA confirmed, in these tissues, by in situ hybridization (ISH) using a mixture of DNA probes for 14 HPV types commonly found in anogenital biopsies (types 6, 11, 16, 18, 31, 33, 35, 42–45, 51, 52, 56) (HPV Omniprobe Set; Digene Diagnostics, Silver Spring, Md., USA) at low stringency conditions. Fourteen cases of SILs, negative for HPV DNA by ISH and polymerase chain reaction (PCR) with consensus primers, were also included in our study. Haematoxylin and eosin-stained sections were reviewed to confirm the histopathological diagnosis. These biopsies were diagnosed as follows: cellular changes characteristic of condyloma and/or mild dysplasia (CIN I) were classified as low-grade SILs (34 cases), and moderate (CIN II) and severe dysplasia-carcinoma in situ (CIN III) were classified as high-grade SILs (29 cases). As controls, 18 cases of normal cervical tissue (including 7 cases of cervicitis) and 13 cases of mature and immature metaplasia, negative for HPV DNA by ISH, were also analysed. To exclude HPV DNA in these tissues, PCR was also performed with consensus primers.

For immunocytochemistry, 5-μm tissue sections were deparaffinized, rehydrated and incubated with 0.05% trypsin (Gibco BRL, Gaithersburg, Mo., USA) or 0.05% pronase E (Sigma, St. Louis, Mo., USA) in TRIS-buffered saline (TBS) for 15 min at 37°C (Table 1). After washings in TBS, the sections were incubated with the primary antibodies, chosen as listed in table 1. After another washing step in TBS, the bound antibodies were localized by the avidin-biotin complex (ABC) method using fuchsin as chromogenic substrate. The sections were finally counterstained with Mayer's haematoxylin and mounted for light microscopy. The immunostaining was evaluated by counting the number of positive cells under light microscopy with ×250 magnification. The cell counts were expressed as numbers of positive cells/0.1 mm^2 sectional area (measured with an image analyser (IBAS 2000; Kontron Bildanalyse, Eching/Munich, Germany) following a method previously described [26, 27]). For the statistical analysis of the results, the Mann-Whitney test or the Kruskal-Wallis non-parametric ANOVA test followed by the Dunnet's multiple comparisons test were applied by using the Instat Mac 2.01 software (GraphPad Software, San Diego, Calif., USA).

ISH was performed as previously described [24, 25, 55, 57]. Tissue sections were first analysed for HPV DNA using the HPV Omniprobe Set (Digene Diagnostics) at low stringency conditions. The specific HPV type was then determined by reanalyzing the tissues at high stringency (T_m –10°C) with HPV 6/11, 16/18 and 31/33/35 biotinylated probe cocktails (ViraType in situ kit, Digene Diagnostics) and individual digoxigenin-labelled probes [25].

To test the possible presence of HPV DNA in histologically normal cervical tissues, PCR was performed using previously described protocols [24, 25, 56, 57]. The primers employed were consensus primers from the L1 regions that have been documented to be capable of amplifying DNA from at least 27 different HPV types, including most of the types that have been isolated from genital tract lesions [71].

Results

Ninety-four cervical biopsies spanning the spectrum from normal epithelium to high-grade SILs were examined for the presence of intraepithelial Langerhans cells and subpopulations of stromal macrophages/dendrocytes by immunohistochemistry using anti-S100, -L1, -CD68 and -factor XIIIa antibodies. The results are shown in Fig. 1 and Table 2.

The density of S100-positive cells was highest in the epithelium of normal biopsies. Most of these cells were distributed within the basal and intermediate layers of epithelium and had a dendritic shape. There was a significant decrease in the density of S100-positive cells in SILs, compared with normal epithelium ($P<0.001$). In addition, S100-positive cells tended to become round with short and non-branched dendrites. Tissues with ma-

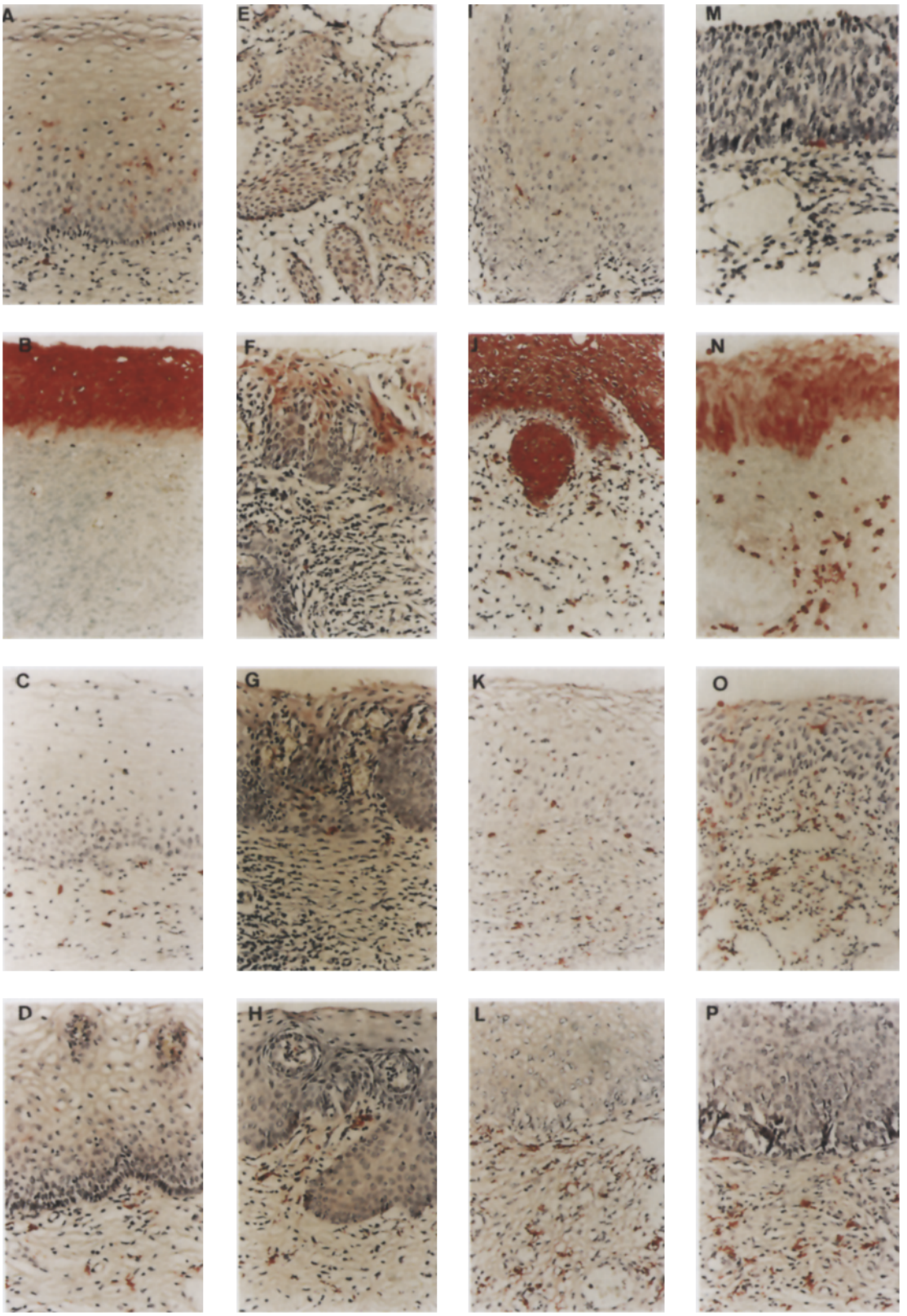

Fig. 1 Representative examples of immunostaining of formalin-fixed paraffin-embedded sections from normal cervical biopsies (**a–d**), cervical biopsies with squamous metaplasia (**e–h**), low-grade squamous intraepithelial lesions (SILs) (**i–l**) and high-grade SILs (**m–p**), after labelling with anti-S100 (**a, e, i, m**), -L1 (**b, f, j, n**), -CD68 (**c, g, k, o**) and -factor XIIIa (**d, h, l, p**) antibodies (×250)

Table 2 Correlation between the histological diagnosis and the density of intraepithelial S100-, stromal Mac 387-, CD68- and factor XIIIa-positive cells (+ cells/0.1 mm^2)

Histology	No. of cases	Density of positive cells[a]			
		S100	L1	CD68	factor XIIIa
Normal cervix	18	35 (13)	1 (1)	16 (13)	40 (11)
Squamous metaplasia	13	7 (4)	2 (2)	6 (9)	23 (9)
Low-grade SILs	34	8 (4)	11 (6)	42 (44)	51 (22)
High-grade SILs	29	8 (7)	19 (8)	112 (51)	55 (15)

[a] Mean (SD); * $P<0.05$; ** $P<0.01$; *** $P<0.001$

Table 3 Correlation between the human papillomavirus (*HPV*) types and the density of intraepithelial S100-, stromal Mac 387-, CD68- and factor XIIIa-positive cells (+ cells/0.1 mm^2) in squamous intraepithelial lesions (*SILs*) separated into low-grade and high-grade

Histology and HPV types	No. of cases (%)[b]	Density of positive cells[a]			
		S100	L1	CD68	Factor XIIIa
Low-grade SILs:	34				
HPV 6/11	7 (26)	11 (3)	11 (4)	32 (33)	46 (12)
HPV 16/18	5 (19)	7 (3)	12 (3)	71 (58)	61 (34)
HPV 31/33/35/novel types	15 (55)	8 (3)	12 (8)	42 (52)	59 (21)
HPV-	7	8 (3)	8 (4)	30 (11)	34 (7)
High-grade SILs	29				
HPV 6/11	0 (0)	–	–	–	–
HVP 16/18	13 (59)	11 (9)	18 (6)	103 (50)	50 (10)
HPV 31/33/35/novel types	9 (41)	9 (4)	23 (11)	128 (67)	59 (19)
HPV-	7	4 (3)	15 (4)	111 (20)	58 (15)

[a] Mean (SD)
[b] Percentage of HPV types in HPV-positive SILs according to their grade

ture or immature metaplasia were also characterized by a decrease in the density of S100-positive cells.

L1-positive cells were rarely encountered in the stroma of normal and metaplastic cervical biopsies. Their density was significantly higher in SILs when compared with normal or metaplastic cervix. CD68- and factor XIIIa-positive cells were detected significantly more often in the stroma of normal and SIL biopsies than L1-positive cells ($P<0.05$ to $P<0.001$). A significantly higher density of CD68-positive cells was observed in high-grade SILs compared with normal or metaplastic biopsies. The density of factor XIIIa-positive cells was found to be higher in SILs compared with metaplastic tissues and in high-grade SILs compared with normal cervical biopsies. The densities of S100- and factor XIIIa-positive cells in low-grade SILs did not differ significantly from those observed in high-grade SILs but CD68- and L1-positive cells were significantly increased in high-grade SILs compared with low-grade SILs. Among normal biopsies, chronic or acute inflammation was not found to be associated with significantly higher densities of stromal L1-, CD68- and factor XIIIa-positive cells (data not shown).

Finally, an attempt was made to correlate the densities of positive-cells for these markers and the HPV type detected in the SILs which were separated into low grade and high grade to look for a relationship independent of the histological grade. HPV types 6/11 were found exclusively in low-grade lesions. HPV 16/18 were present in more than 50% of the high-grade lesions. Types 31/33/35 and novel types were the most frequent HPVs detected in our series of SILs. They were represented in approximately equal percentages in both low-grade and high-grade lesions. We did not find any specific relationship between the density of S100-, L1-, CD68-, factor XIIIa-positive cells and the HPV type (Table 3). There was no significant difference between the densities of immunostained cells in HPV-positive and HPV-negative SILs. HPV DNA was not detected, even by PCR, in the histologically normal or metaplastic cervical tissues which were tested in the present work. All positive and negative controls for the ISH and PCR techniques gave the expected results.

Discussion

The strong association between HPV infection and genital neoplasia has prompted a variety of studies designed to evaluate the host immune response to this potentially oncogenic virus, both from the perspective of identifying patients at risk as well as defining immunological responses that may prevent the disease.

Langerhans' cells are derived from the bone marrow and form a network in squamous epithelia throughout the body. These cells play a pivotal role in immune surveil-

lance against cutaneous and mucosal viral infections and neoplasms due to their capacity to capture and present antigens and induce a specific T-cell activation [10, 20, 49]. Our data are congruent with those from other studies, reporting a depletion and morphological alterations of Langerhans' cells in cervical intraepithelial lesions [20, 48, 51, 74, 77]. We also noted a significant decrease in the density of S100-positive Langerhans' cells in metaplastic epithelium compared with normal exocervical tissue. The reduction of Langerhans' cells in immature metaplasia has already been reported [30] and it has been suggested that the presence of glandular columnar cells provides some kind of negative chemotactic stimulus which prevents migration of Langerhans' cells into metaplastic epithelium [30]. The density of Langerhans' cells detected by anti-S100 antibody in the present study was similar to that previously reported with other antibodies, such as anti-CD1a, in normal and pathological cervical tissues [51]. A similar depletion of Langerhans' cells has also been observed in HPV-related lesions of other squamous epithelia, even when these develop in the absence of any pre-existing metaplastic process, such as penile condylomata [51], cutaneous warts [19], laryngeal papillomas [18] and skin lesions of epidermodysplasia verruciformis patients [35], suggesting that HPV infection by itself is a main factor in the depletion of Langerhans' cells in SILs. It has been demonstrated that more than 90% of cervical SILs are HPV-associated [25, 44, 76] and that, with sufficiently sensitive diagnostic methods, HPV DNA can be detected in most preneoplastic cervical lesions. Some studies using Southern blot hybridization or PCR have, however, demonstrated that a minority of SILs and invasive carcinomas did not contain detectable HPV DNA sequences, suggesting that some cervical neoplastic lesions develop independently of HPV infection [9, 66]. Fourteen of these cases (7 low-grade and 7 high-grade SILs), in which HPV DNA was not detected by ISH and PCR with consensus primers, were tested in our study for the presence of intraepithelial Langerhans' cells and subpopulations of stromal macrophages/dendrocytes. There was no significant difference between the densities of these cells in HPV-positive and HPV-negative SILs, suggesting that the modifications in the density of Langerhans' cells and macrophages/dendrocytes observed in SILs may also be associated with the preneoplastic process initiated by other oncogenic agents. Although the link between HPV infection and cervical SILs or invasive neoplasia is well established, other carcinogens, such as radiation, tobacco products or hormonal factors, have been considered to play a part in the development of keratinocytic malignancies [84]. Whether these factors promote cervical carcinogenesis by acting directly on keratinocytes or by inducing modifications of local cellular immunity is still unclear. For example, tobacco smoking over a long period may be a direct carcinogenic factor and may produce a local immunosuppressive effect. High concentrations of cigarette smoke constituents, such as nicotine, have been identified in cervical mucus [70] and cigarette smoking has been shown to be associated with a significant decrease in the Langerhans' cell population in both normal and preneoplastic cervical epithelium [8]. The combined action of tobacco and oral contraception has also been reported to interfere with the metabolism of vitamin A, the role of which has been demonstrated in cell differentiation and immunity [59].

For HPV-associated SILs, some authors have suggested that the Langerhans' cell changes might be due to the cytopathic effect of the virus [52]. However, the restricted epithelial cytotropism of HPV and the lack of evidence for Langerhans' cell infection make this hypothesis unlikely. Alterations of membrane molecules of infected keratinocytes (loss of HLA class 1 antigen or E-cadherin) might contribute to the reduction of Langerhans' cells or to their functional inability [20, 73]. Recently, keratinocytes have been shown to produce immunoregulatory cytokines that are likely to have a stimulatory effect on Langerhans' cells, such as tumour necrosis factor alpha (TNFα) and granulocyte-macrophage colony-stimulating-factor [13, 54, 82]. HPV infection of the keratinocytes has been shown to alter this function [82], causing a reduction of cytokine production that may lead to a decreased stimulation of Langerhans' cells. Another possibility is that the immunosuppressive cytokine interleukin-10, which is known to be expressed in SILs (Crowley-Nowick, personal communication) and in UV-irradiated keratinocytes [67], plays a role in the depletion or inhibition of Langerhans' cells.

Whatever the mechanism by which the density of intraepithelial Langerhans' cells is reduced in SILs, this alteration might result in a locally acquired immunodeficiency that allows the HPV to persist in the cervix and exert a neoplastic transforming effect.

The density of stromal cells immunostained with antibodies against macrophage/dendrocyte-associated antigens was modulated inversely to that of intraepithelial Langerhans' cells in normal biopsies and HPV-infected cervical tissues. The densities of L1-, CD68- and factor XIIIa-positive cells were increased in the stroma underlying SILs compared with normal or metaplastic cervical tissues. These data are in agreement with other studies reporting a macrophage/dendrocyte infiltration in preneoplastic [75] and neoplastic [39] cervical lesions as well as in anogenital warts [32] and tumours from other sites [2, 5, 6, 29]. The presence of infiltrating macrophages/dendrocytes may be related to aspecific stimuli, such as the release of chemoattractants due to local inflammation or cell death or due to cytokine production. A recent study showed that TNFα strongly induces the expression of the *JE* gene, encoding the monocyte chemoattractant protein (MCP-1), in non-tumorigenic somatic cell hybrids of HPV-transformed keratinocytes with normal human fibroblasts and that the expression of this protein is accompanied by a dose-dependent reduction of HPV transcription [68]. Interestingly, the MCP-1 protein has been shown not only to stimulate the monocyte migration but also to activate monocytes to secrete a variety of different cytokines [47, 50] which can exert

different biological effects on the target cells. These cytokines, including TNFα, produced by activated monocytes, may induce the secretion of larger amounts of the MCP-1 protein by other mononuclear cells or keratinocytes, leading to higher monocyte migration. As regression of HPV-induced lesions is a frequent event and has been shown to be accompanied by a local infiltration of inflammatory cells, including macrophages [1, 32, 58], the increased density of stromal macrophage antigen-positive cells may also be an early feature of regressing viral lesions. Although the mechanisms of recognition and killing are not well understood, previous studies have shown that activated macrophages are able to kill cells transformed by HPV 16 [7, 28] and other DNA tumour viruses [17, 22] selectively. In the in vitro studies of Denis et al. [28], the killing of HPV-transformed cells was only observed with activated macrophages and was contact dependent, two conditions that are not necessarily always present in vivo. We have shown, however, that TNFα, a macrophage and dendrocyte-derived growth-inhibitory cytokine [53], when associated with interferon gamma, exerted a marked antiproliferative effect on HPV-transformed keratinocytes grown on collagen rafts as well as in monolayer cultures [26]. Although the increased density of macrophage antigen-positive cell populations may reflect a direct anti-tumour activity against SILs, the macrophages may also have a symbiotic relationship with (pre)neoplastic cells by producing growth factors, such as transforming growth factor beta, and having angiogenetic activity [47]. Other studies have suggested that activated macrophages might promote neoplastic progression by inducing gene alterations [33, 80, 81].

The HPV type distribution described in this study is in agreement with several other reports [4, 25, 45, 57]. No specific relationships were observed between the density of intraepithelial Langerhans' cells or stromal macrophages and the HPV types detected in SILs separated into low and high grade.

We have shown that intraepithelial Langerhans' cells and stromal macrophages were inversely modulated in normal and SIL biopsies. A local intraepithelial immunodeficiency based on a depletion of Langerhans' cells has been postulated as a mechanism by which HPV could be involved in the genesis of carcinoma [20, 51, 74]. Further studies are needed to investigate the mechanism responsible for the decreased density of Langerhans' cells in SILs and the functional role of these cells. Whether the increased macrophage stromal infiltration in SIL biopsies reflects ongoing cellular immune mechanisms in the host defence against virally infected preneoplastic cells, a non-specific role of macrophages or a symbiotic relationship with preneoplastic cells also requires further elucidation.

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