Rev. Bras. Cancerol., 1999; 45(1): 09 - 18

Configuração da superfície das células epiteliais infectadas pelo papilomavírus humano

Configuration of the surfaces of epithelial cells infected by human papillomavirus

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Resumo

Tipos de papilomavírus humano foram investigados por hibridização in vitro, por meio de biópsias realizadas em 18 pacientes com diversas patologias do colo do útero (7 condilomas, 1 NIC I + HPV, 3 NIC II, 4 NIC II + HPV e 3 NIC III). Esfregaços cervicais dos mesmos pacientes foram processados por microscopia de elétrons, para se estudar a configuração das superfícies de células infectadas por diferentes tipos de HPV.

Sete condilomas, 1 NIC I + HPV, 3 NIC II e 3 NIC II + HPV mostraram configurações de superfície quase idênticas àquelas de células normais (curtas, finas microvilosidades homogêneas ou microfibras típicas). Este grupo apresentou baixos, moderados e altos riscos para tipos de HPV em forma episomal, ao passo que 1 NIC II + HPV e 3 NIC III com HPV 16/18 integrados apresentaram células cobertas com microvilosidade macroscópica, de tamanho variável e distribuição desigual, ou uma superfície amorfa.

A presença de HPV 16/18 não é bastante para o desenvolvimento de uma configuração anormal, mas o estado integrado deste tipo viral pode estar relacionado a alterações morfológicas da superfície celular.

Os resultados deste trabalho ressaltam a importância da integração viral na gênese do carcinoma cervical.

Palavras-chave: papilomavírus humano; neoplasia intra-epitelial cervical; microscopia eletrônica; hibridização *in situ*

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This paper was presented in part at the X Congreso Argentino de Citología, Buenos Aires, Argentina, 6 / 9 December, 1992.

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Abstract

Human papillomavirus (HPV) types were investigated by in situ hybridization (ISH) in biopsies from 18 patients with diverse pathologies of cervix uteri (7 condylomas, 1 CIN I + HPV, 3 CIN II, 4 CIN II + HPV and 3 CIN III). Cervical smears of the same patients were processed by Scanning Electron Mycroscopy (SEM) in order to study the configuration of surfaces of cells infected by different HPV types.

Seven condylomas, 1 CIN I + HPV, 3 CIN II and 3 CIN II + HPV showed almost identical surface configurations to those of normal cells (short, thin, homogeneous microvilli *or typical* microridges*). This group presented low, moderate and high risk HPV types in a episomal form, while 1 CIN II + HPV and 3 CIN III with integrated HPV 16/18 exfoliated cells covered with gross* microvilli, *with variable length and uneven distribution, or an amorphous surface. The presence of HPV 16/18 cannot be sufficient for the development of an anormal configuration, but the integrated state of this viral type could be related to the morphological changes in cellular surface morphology.*

The results of this paper underline the importance of viral integration in the genesis of cervical carcinoma.

Key words: *human papillomavirus; cervical intraepithelial neoplasia; scanning electron microscopy; hybridization in situ.*

Introduction

Cervical carcinoma evolves from a spectrum of premalignant intraepithelial lesions (cervical intraepithelial neoplasia: CIN)[1]. Long-term follow-up of these lesions indicate a rise of relative risk from CIN I to CIN III[2, 3, 4].

The process of neoplastic transformation is accompanied by in vitro phenotypic changes, like altered cellular morphology, expression of new products and surface antigens or alterations in cytoskeletal and membrane architecture[5]. In some cases, the modification of cell surfaces could be studied in vivo by means of Scanning Electron Microscopy (SEM).

Experimental evidence supports a pathogenic role of Human Papillomavirus (HPV) in cervical neoplasia[6]: early and late viral antigens have been detected in CIN[7, 8], and HPV-DNA is integrated into the host genome in CIN[9], immortalized keratinocytes[10], cell lines from cervical cancers[11] and squamous cervical carcinomas[12]. Active transcription and translation of the E6-E7 region of HPV 16 or HPV 18 have been showed in cell lines derived from cervical cancers[13, 14]. The E6 and E7 proteins of these high-risk viruses form complexes with p53 and pRb, respectively[15, 16], both proteins encoded by host suppressor genes, which are associated with regulation of cell proliferation and differentiation[17].

However, the presence and integration of HPVs, even when necessary, cannot be sufficient for malignant conversion, since a low number of infected individuals eventually develop cancer. Intracellular and intercellular systems have been proposed in order to protect the host by suppressing viral oncogene transcription or by post-transcriptional control of viral oncogene function[18].This study was undertaken to investigate changes in cellular surfaces of cells exfoliated from cervix uteri, infected by different HPV types.

Materials and methods

Eighteen patients with diverses pathologies of cervix uteri were selected (7 condylomas, 1 CIN I + HPV, 3 CIN II, 4 CIN II + HPV and 3 CIN III), and biopsies were performed in order to investigate HPV types by ISH (Pathogene-Enzo diagnostic), according to the following protocol: Deparaffination in xylene, hydration, treatment with Proteinase K, endogenous peroxidase blockade, dehydration, DNA denaturalization and hybridization with biotinylated probes (6/11, 31/33/35 , 16/18 HPV), post-hybridization reactive, detection with streptavidin-biotinylated horseradish peroxidase complex and viewed with 3-amino-9-ethylcarbazole (AEC) and hydrogen peroxide[19].

The SiHa (HPV 16) and HeLa (HPV 18) cell lines, and cervical biopsies with different proved HPV types were used as controls. The physical state of the viral genome was determined by classifying the signals into three types: 1: *Diffuse*, 2: *punctuate dots* and 3: *combined 1 and 2.*[20].

Cervical smears in celluloid for SEM studies were obtained. The smears were fixed in 2.5%

glutaraldehyde + 1% sodium cacodylate in phosphate buffer pH 7.2, stained with Papanicolaou technique, post-fixed with 1% osmium tetroxide, dehydrated in acetone, dried by critical point (Balzers CPD 030), paladium-platinum sputtered and observed through a Scanning Electron Microscopes (JEOL-JSM-35 CF and ECON IV-Philips)[21].

Normal cells exfoliated from different layers of cervix uteri epithelium were observed for control. The cellular surfaces observed by SEM were classified into two classes:

a) *Regular pattern*: short, thin, homogeneous microvilli or typical microridges.

b) *Irregular pattern*: gross microvillie, with variable length and uneven distribution, or an amorphous surface.

Investigation of HPV' late protein was carried out in the biopsies and cytological smears of all patients, using the Sternberger technique[22]. Cervical smears (processed by Papanicolaou technique) were destained and the immune peroxidase indirect method was applied according to the following protocol: endogenous peroxidase blockade, inespecific antigens blockade, incubation with policlonal antibody anti-L2 (Dako and Biogenex), with link antibody and finally with peroxidase-antiperoxidase complex (Biogenex).

The peroxidase enzime was visualized using 3,3 Diamino benzidine tetrahydrochloride in buffer Tris pH 7.6 and Hydrogen peroxide. The same technique was used in biopsies, after they were deparaffinized and rehydrated.

Results

In all low-grade lesions (CIN I + HPV, condylomas), the HPV types detected were 6/11 (low risk types). The high risk types (16/18) and the intermediate risk HPV types (31/33/35) were detected in CIN II, CIN II + HPV and CIN III.

The HIS signal type 1 was observed in low-grade lesions, in 3 CIN II and 3 CIN II +HPV (Figure 1).

All CIN III showed signals type 2 or 3, while 1 CIN II + HPV presented the combined type 3 (Figure 2).

A regular pattern of cellular surfaces were observed by SEM in the cells exfoliated from condylomas, CIN I + HPV, CIN II (Figures 3, 4) and most of CIN II + HPV, while CIN III (Figure 5) and one of the CIN II + HPV (Figure 6) presented an irregular pattern (Table 1).

The HPV' capsid antigen was revealed in the upper layers of the cervical biopsies and

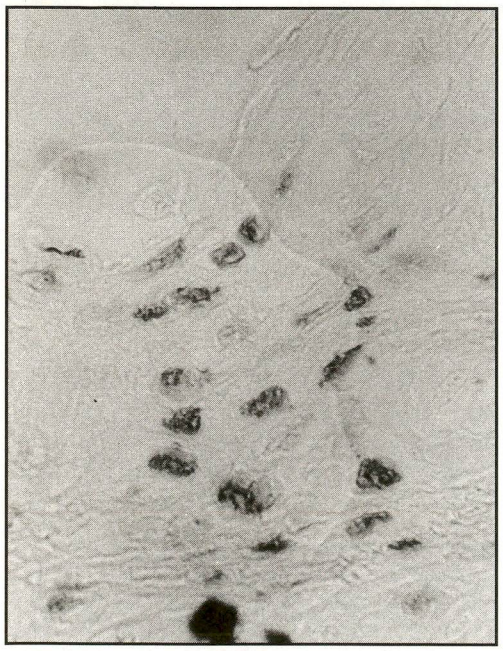


Figure 1 - Hybridization in situ: Episomal HPV 6/11 in a biopsy from condyloma of the cervix uteri (Signal type 1) (X 500).

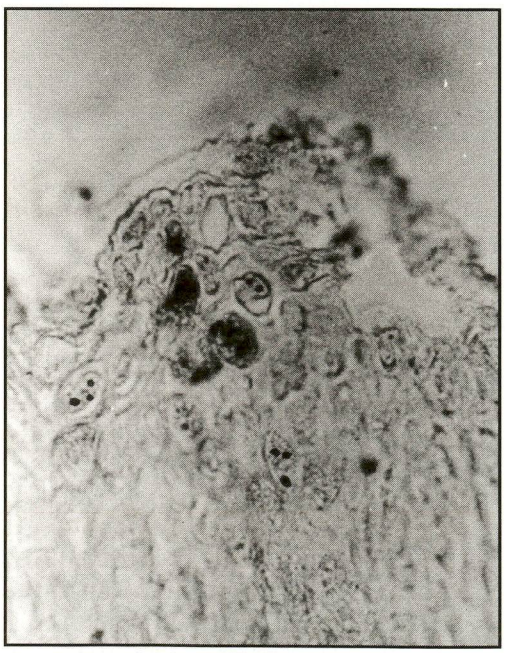


Figure 2 - Hybridization in situ: Signal type 3 for HPV 16/18 in a biopsy from CIN III of the cervix uteri (X 500).

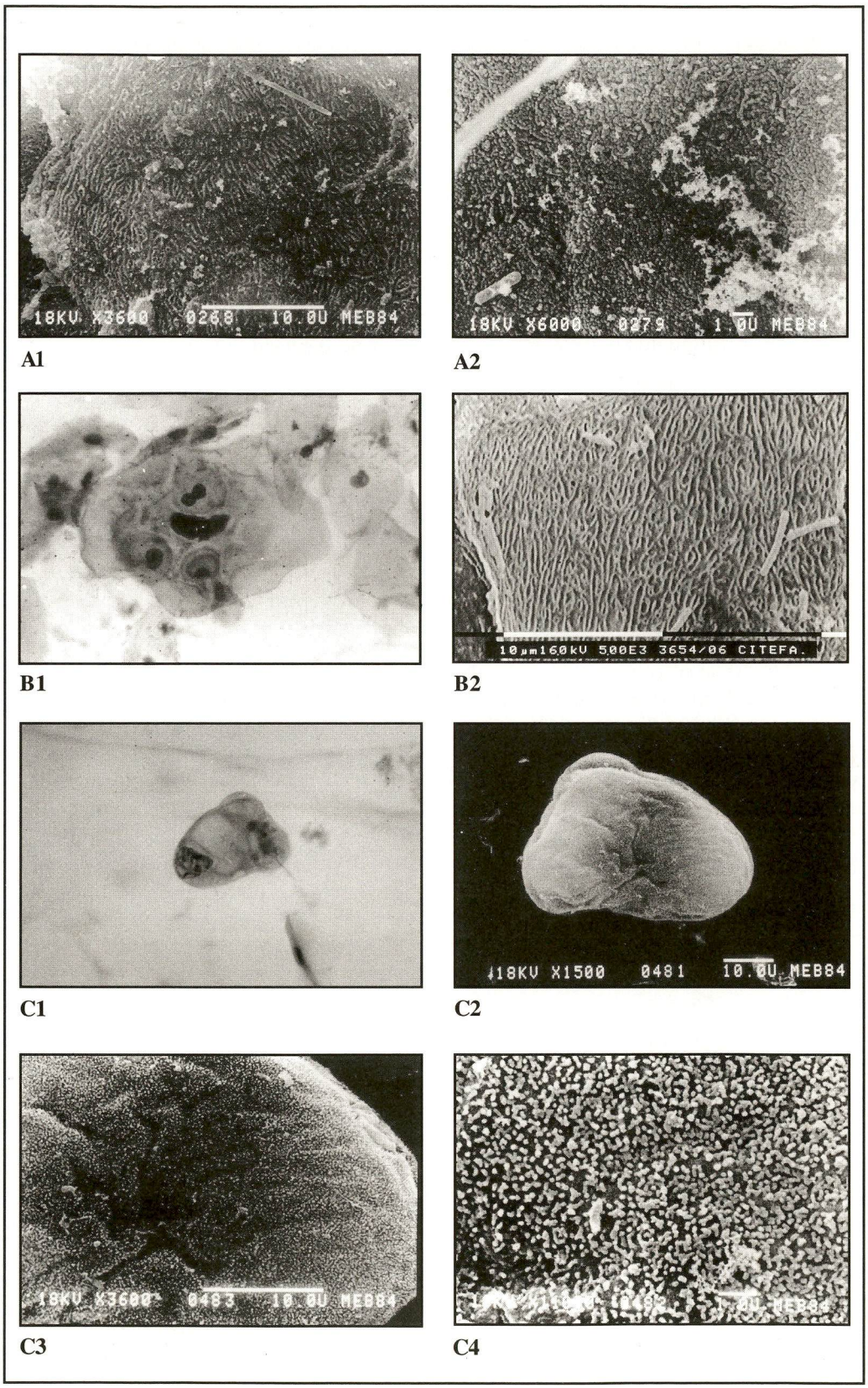
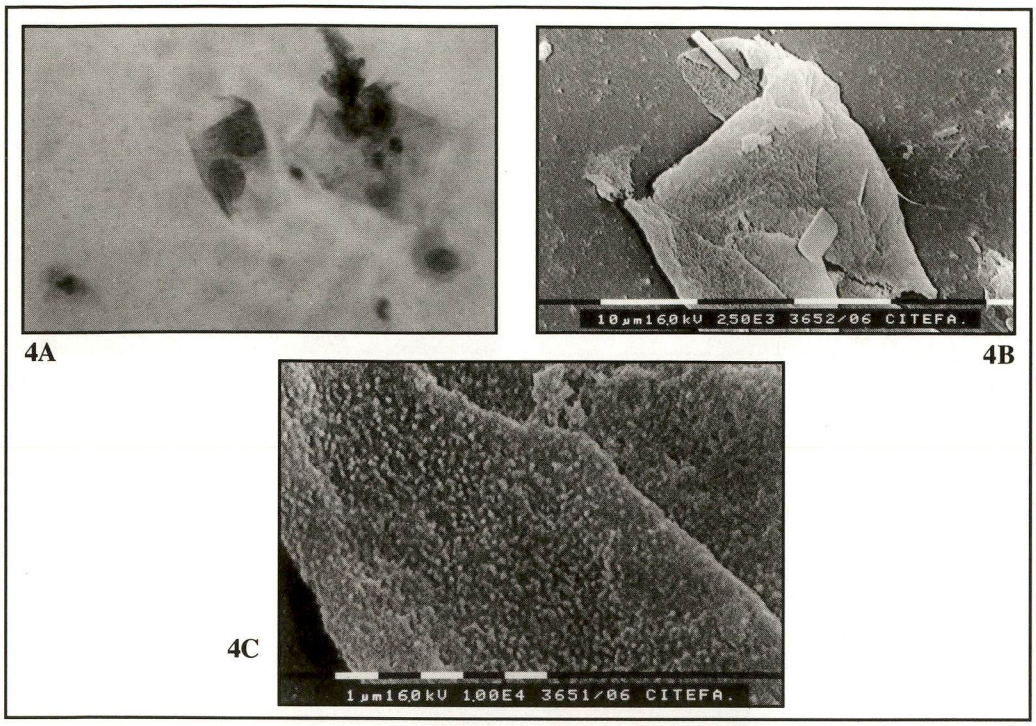


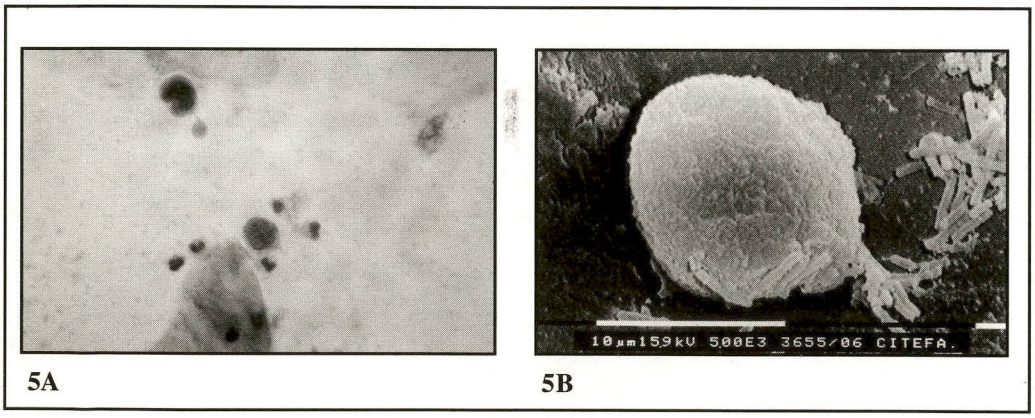
Figure 3 - A) SEM of exfoliated cells from cervix uteri: normal cellular surfaces showing microridges in a superficial cell (A1) and microvillie in a intermediate cell (A2) (X 3600 and X 6000 respectively). B) SEM of a superficial cell with dyskeratotic inclusions from a cervix uteri infected by HPV 6/11: regular pattern of microridges. (B1: Papanicolaou stain X 500; B2: SEM X 6500). C) Exfoliated cell from a cervix uteri infected by HPV 6/11: regular pattern of microvillie (C1: Papanicolaou stain X500; C2, C3 and C4: SEM X 1500, 3600 and 11000 respectively).



4A 4B

4C

Figure 4 - Exfoliated cells from CIN II of cervix uteri (HPV 16/18) showing regular pattern by SEM (4A: Papanicolaou stain X 500; 4B and 4C: SEM X 3200 and 12000 respectively).

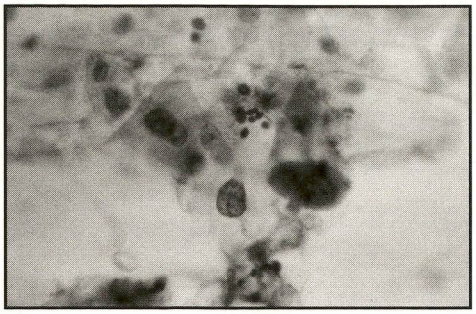


5A 5B

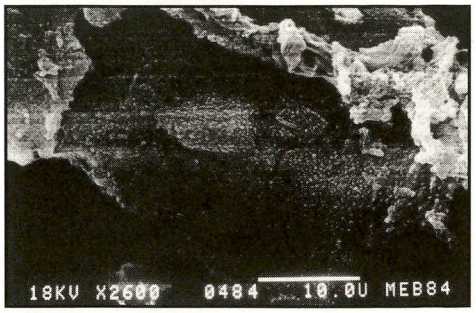
Figure 5 - CIN III of a cervix uteri (HPV 16/18).5A: Exfoliated cells stained by Papanicolaou method (X 500). 5B: SEM of one of the cells showed in 5A with irregular pattern (X 6500).

Table 1 - Comparison between ISH (biopsies) and cellular surface patterns by SEM (cervical smears).

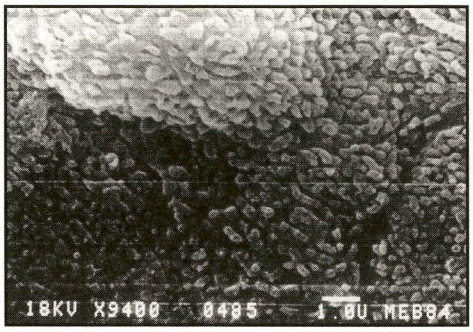
	HPV types	Signal types	Surface patterns
Condyloma (n=7)	6/11	1	Regular
CIN I + HPV(n=1)	6/11	1	Regular
CNI II (n=3)	16/18	1	Regular
CIN II + HPV (n=3)	16/18-31/33/35	1	Regular
CIN II + HPV (n=1)	16/18	3	Irregular
CIN III (n=3)	16/18	2-3	Irregular



6A



6B



6C

Figure 6 - CIN II + HPV of a cervix uteri (HPV 16/18). 6A: Papanicolaou stain (X 500). 6B and 6C: SEM of one of the cells showed in 6A dysplaing an incipient irregular pattern (X 2600 and 9400 respectively).

Table 2 - Investigation of capsid antigen in biopsies and cervical smears.

Condylomas	7/7
CIN I +HPV	1/1
CIN II	3/3
CIN II + HPV	2/4
CIN III	0/3

(Positive/total)

in the cells exfoliated from them in the condylomas, CIN I +HPV, CIN II and in 2 cases of CIN II + HPV. No reaction was observed in CIN III and in 2 cases of CIN II + HPV (Table 2).

Discussion

In the molecular mechanism of HPV-associated carcinogenesis, the integration of HPV-DNA into the host genome plays an important role: the consequence of the disruption of E1/E2 open reading frames (OPR) is the overexpression of E6 and E7 ORF[23-25].

The HPV-DNA integration, considered as one of the first steps in the multistage development of human cervical cancer, has been demonstrated in cell lines from human cervical cancers, cervical intraepithelial neoplasia (CIN), immortalised human keratinocytes and squamous cervical cancers[9-12].

Although in benign HPV-induced genital lesions and in low grade CIN the viral genomes are predominantly episomes and 6/11 types[26], integration of HPV genomes of high risk HPV types can take place very early in

the cervical cancer development, but can persist in this state without transcriptional activity[27]. Some models attempt to explain the suppression of E6/E7 in vivo, postulating the existence of a cellular interfering factor (CIF)[28] and the regulation by an intercellular interaction (macrophages and other cell types)[29]. Changes in cellular surfaces were reported in cervical cytology[21,30-32], urothelium[33] and effusions[34,35] in presence of cancer. This paper tries to relate the physical state of the viral genome to the modifications in the surfaces of cells exfoliated from condylomas and CIN. All the benign HPV-induced lesions and almost the totality of CIN II showed signals type 1 by ISH, indicating that these cervical biopsies contained episomal forms, even when CIN II presented high risk HPV types (16/18). Some groups have reported high percentages of HPV-DNA integration in CIN[27], while others showed that detectable integration occurs infrequently in CIN[12]. The cells exfoliated from this group of lesions presented surface configurations almost identical to those of normal control cells.

The upper surfaces of normal superficial cells are covered with microridges and the lower surfaces (facing towards the deep layers) are covered with microvillies[21]. This distribution is maintained in superficial cells exfoliated from benign HPV-induced lesions: depending on the surface leant on the slide, it is possible to see both structures, while in the intermediate type and deep-layer type of cells arising from CIN II with signal type 1 by SEM, only homogeneous microvillies were observed.

Typical koilocytes from the low-grade lesions presented the characteristic depression corresponding to the perinuclear halo, with regular pattern in this area. Outside the halo, the cell showed amorphous and wrinkled surface, as a consequence of keratinization processes. Signal types 2 and 3 were observed in CIN III and in one case of CIN II + HPV. SEM of exfoliated cells from this group showed an irregular pattern. Wherefore, the integrated state of HVP-DNA could be in relation to the morphological changes in cellular surfaces observed in these pathologies. The biologic behavior of these lesions were not informed in this paper; however, it is important to point out that the case with CIN II + HPV and irregular pattern developed a CIN III six months later, while most of women having pathologies with regular pattern (5 condylomas and 2 CIN II with HPV 16/18) returned in six to eigth months.

Epithelial cells which do not allow viral replication and productive infection (capsid formation) may be transformed by HPV, which can become integrated to the host genome[36]. This mechanism is favored in low-differentiated epithelium, as in CIN III, where L2 and L1 transcripts do no exist. In our assay for capsid antigen of HPV, it was positive in terminally differentiated keratinocytes of low-grade lesions and in about half of CIN II (these showing cellular differentiation); in the majority of high-grade CIN, with no-permissive cells and more possibility for HPV-DNA integration, the HPV capsid was not detected. Although working with a small number of cases, the data presented in this paper could support the fact that the episomal state of HPV-DNA does not modify the normal architecture of cellular surfaces, while the integration of high risk HPV types could be associated to an irregular pattern. Induction of aneuploidy by HPV 16 could lead to activation of oncogenes[37], and this phenomenon could interfere with the stabilization of cytoskeletal structures, like those related to the microridges or to the size and shape of microvillies[38]. However, the presence of HPV 16/18 cannot be enough for the development of an irregular pattern, since 3 cases of CIN II and 3 cases of CIN II + HPV with types 16/18 and 31/33/35 showed regular patterns. Even when HPV-DNA is integrated to the host DNA, the expression of E6/E7 ORF may be suppressed by intra and intercellular factors. High risk HPV types are considered precursors of CIN and cervical carcinomas, but they are not sufficient for the malignant transformation. Different co-factors like cigarette smoking[39], hormones[40], herpes[41] and changes in local cervical immunology seem to be related to the etiology of cervical neoplasia[42].

If alterations in membrane architecture accompany the process of neoplastic transformation[5,43], the observation of infected cells showing abnormal surfaces, with integrated high risk HPV, support the mechanism of viral integration as an important step in the development of cervical neoplasia.

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