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Full Length Research Paper

Ultrastructural changes in premalignant and malignant lesions of the uterine cervix with papillomavirus infection

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The main purpose of this study was intended to determine the possible ultrastructural relations among the human papilloma virus infection, angiogenesis and cellular infiltrate in premalignant and malignant lesions of uterine cervix. 58 samples of cervix were obtained from patients of the ambulatory "Maria Teresa Toro" Aragua, Venezuela, with cervical intraepithelial neoplasia (CIN) and human papillomavirus (HPV) infection. For the ultrastructural study, routine transmission electron microscopy techniques were used as well as detection and type definition of HPV by PCR. At the ultrastructural level in CIN I samples, vessels reduplication of basement membrane was founded. In this case, type 6 was detected in all CIN samples. In CIN II samples, with a positive report of HPV, 16 numerous mast cells were observed. A sample with a diagnosis of CIN III and HPV16 exhibited mast cell invaginating toward capillary lumen. In a sample of invasive cervix carcinoma, HPV11 was detected, endothelial cytoplasm prolongations into the lumen were found and the endothelial wall was widened. Changes of microvasculature tissue in premalignant lesions and an increase of thickness of the endothelial wall of capillaries in malignant lesions of uterine cervix indicated the activation of the angiogenesis. Mast cells in the vascular periphery, not only in malignant lesions, but also in premalignant lesions indicated its relationship with angiogenic processes.

Key words: Cellular infiltrate, endothelial vascular, cervical cancer, HVP, ultrastructure.

INTRODUCTION

Tumor angiogenesis is a necessary step required for the transition from a small and harmless group of tumor cells to a malignant tumor of large size (Lozano, 2000). Neov-ascularization is induced by the persistence of blood vessels intimately associated with the proliferation, in-vasion and metastasis of solid tumors (Ichiro et al., 2003; Ross et al., 2005). It is related to an ample variety of growth factors produced by neoplastic inflammatory cells and endothelial cells. The association of angiogenesis with the biological histological behavior of tumor cells isinfiltrated in a tumor tissue. In other instances, where macrophages are not sufficiently clear, there is a debate on tumor vascularity, decisive evidence (which is on the final decision on histolo-gical type of tumor) and the degree of differentiation of tumor cells. The endothelial cell has an important role in blood homeostasis, while its functional properties change in response to diverse stimulus. This process is known as endothelial activation and is also responsible for the pathogenesis-etiology of many vascular pathologies. In-ducers of endothelial activity are considered bacterial, viral and cytotoxic agents as well as components of com-plement, lipidic products and hypoxia (Ross et al., 2005; Tonino et al., 2001). Lately, a trial has been made on the correlation bet-ween vascular density as a measure of angiogenic res-ponse and tumor malignancy, since prognosis aims in mammary, ovary, prostate and colon cancers (Ross et al., 2005; Vidal and Horw, 2002). A technique which allows evaluating vascular tissue in premalignant lesions

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Abbreviations: CIN, Cervical intraepithelial neoplasia; **HPV,** human papillomavirus; **Aa,** acetic acid.

of uterine cervix is the colposcopy. By using it, biopsy is obtained in a reliable manner, in order the final diagnosis to be made by the histopathology study. Coloposcopic index, based on lesion edges, color, vas-cular patterns and reaction to iodine may improve dif-ferentiation between intraepithelial neoplasia of low grade (CIN I) and more significant degrees (Baillie et al., 1995).

The vascular pattern of CIN I observed by colposcopy is formed by uniform vessels of fine caliber frequently disposed in a loose and random manner as a horizontal network. Additionally, swollen capillary loops may not be present in the vertical path toward the surface with uniform vascular caliber. However, intraepithelial lesions of high caliber (CIN II, III) show an abnormal vasculature. After applying acetic acid (Aa), almost all lesions of CIN III are observed as white spots lacking any vascular pattern which is due to the constriction of such narrow vessels because of the edema induced by the application of Aa. Thereafter, it is possible to observe the classic dots in mosaic, found in a small percentage of high risk lesions. Swelling of vessels of vertical orientation produces a structure disposition of random direction with irregular spirals. Angiogenic factors, produced by high risk lesions, cause the development of prominent swollen conducts that are seen separately by a colposcopy superficial epithelium in a group of individual blocks or mosaic pattern. These patterns increase in width and the intercapillary distance can grow as lesion malignancy augments (Baillie et al., 1995). The extension of vascularity in a determined region of malignant lesion depends on the balance among stimuli and inhibitors of angiogenesis produced by tumor cells (Ichiro et al., 2003; Lorinz and Reid, 1996).

Recently, cellular proliferative activity has been considered as a marker of malignancy potential for diverse carcinomas, demonstrating that there is a narrow relation among tumor angiogenesis, cellular proliferation and clinic results in some studies (Ichiro et al. 2003; Hanahan and Folkman, 1996). Additionally, a fast vascularization of tumor, posterior to reversal of inhibitory therapies of vascular endothelial growth factor (VEGF) (Maeda et al., 1996) has been observed and an association between p53 and VEGF as predictor of tumor vascularization in bladder cancer (Mancuso et al., 2006), due to a cor-relation with tumor angiogenesis, has also been observed. This is important in the formation of new blood vessels, its expression and the consequent microvascular density which has an im-portant role in the tumor growth and metastasis, with a possible value as prognosis index (Tian et al., 2006; Du et al., 2003). Other important element related to malign-nant process is the presence of mast cell because its degranulation plays a significant role in the variety of re-actions of chronic inflammation and neovascularization (Lozano, 2000).

Mast cell arises from progenitor cells located in the bone marrow. However, in normal conditions, there are no circulating mast cells, since the progenitor cells migrate to peripheral tissues as immature cells, differentiating *in situ.* The mature mastocytes are distributed in the whole organism prevailing in the proximities of blood vessels, nerves and epithelium. Mast cell activation originates three types of biological response: Secretion of preformed content of granules by a process of regulated exocytosis, synthesis and secretion of lipid mediators and synthesis and secretion of cytokines. They can also secrete tumor necrosis factor (TNF) (Carusos et al., 1997; Abbas and Lichtman, 2005). This is provoked in vascular epithelial cells adhesion of leukocytes, neutrophils, monocytes and lymphocytes in inflammatory process and induces apoptosis of some cellular types. Mast cells have also been related to the development and progression of basal cell carcinoma, squamous cell carcinoma and melanoma.

Additionally, there are evidences that suggest the intervention of mast cell in the genesis of malignant cutaneous tumors by the activation of vascular endo-thelial growth factor in basal cell carcinoma and melanoma (Nienartowicz et al., 2006; Ch'nh et al., 2006). The present work was intended to determine a possible ultrastructural relation among infection by HPV, angioge-nesis and cellular infiltrate in pre-malignant and malignant lesions of uterine cervix.

MATERIALS AND METHODS

Fifty-eight samples of cervix obtained from uterine cervix pathology clinic patients of María Teresa Toro ambulatory in Aragua state, Venezuela, were studied. At the time of study, all samples had cytological and histopathological reports of infection by human papilloma virus (HPV), without treatment. For the ultrastructural study, pieces of cervix were fixed with 3% glutaraldehyde and 1% OsO4, where both fixatives were diluted in phosphate buffer (pH = 7,4 and 320 mOsm), dehydrated in increasing ethanol concentrations and embedded in EMBed-812 resin (Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections were cut in a Porter-Blum MT2-B ultramicrotome with a diamond knife and were stained with uranyl acetate and lead citrate. Sections were examined with a JEM-1011 transmission electron microscope.

DNA isolation sample preparation

Swabs for cytology and human papillomavirus were obtained. The samples for DNA extraction were suspended in 100 µl of digestion buffer (50 mM Tris- HCL, pH 8.0, 1 mM EDTA and 1% N-laurilsarcosin) containing 0.5 mg/ml of proteinase-K and incubated for 24 h at 55˚C. DNA was purified with phenol- chloroform isoamyl alcohol and precipitated by sal ethanol. The DNA was dissolved in 50 µl of TE buffer.

PCR assay

HPV status was determinated by PCR with the L1 consensus primers MY09 and MY11. This PCR assay was used to detect 27 HPV types known to infect the genital tract in the DNA purified from 58 cervix samples. To determine specimen adequacy, the GH20/ PC04 human β- Globin target was co-amplified with HPV sequences. Each amplification contained 10 mM Tris- HCl (pH 8.5), 5 mM KCl, 4mM $MgCl_2$,a 200µM concentration (each) of dCTP, dGTP, dATP and dTTP, 7 .5 U of AmpliTaq, 2.5 pmol (each) of

Table 1. Lesions premalignant of uterine cervix with infection of HVP.

	Cervical intraepithelial neoplasia (CIN)	
	Case	**%**
CIN I	22	37.90
CIN II	21	36.20
CIN III	9	15.50
Insuf.	6	10.30
Total	58	100

Note: Samples of cervix were obtained in the Uterine Cervix Pathology, Department of the María Teresa Toro Outpatient Clinic in Aragua State, Venezuela.

the B- globin amplification primers and 5 to 10 µ (approximately 500 ng) of template DNA. Reaction was amplified in a MJ Research PTC- 150 thermal cycler by using the following profile: 94ºC for 4 min, 40 cycles of 94ºC for 1 min (denaturation), 55ºC for 1 min (annealing), 72ºC for 1 min (extension), 72ºC for 5 min (final extension) and 4˚C held as the final step. A known positive specimen and a negative (no DNA) specimen were included in each assay as controls.

PCR assay for typing human papillomaviruses

The typing of HPV was determinated by MPCR Amplification/Detections kits. These kits have been designed to direct the simultaneous amplification of specific E6 *gene* of the HPV type - 6, 11, 16, 18 and 33. Each amplification contained 25 µl 2 × MPC Buffer Mixture, 5 µl 10 × MPCR Primers, 0.5 µl Taq DNA polymerase (5 U/µl), 14.5 µl H2O, and 5 - 10 µl of template DNA. Reactions were amplified in a MJ Research PTC- 150 thermal cycler by using the following profile: 2 cycles of 96˚C for 1 min, 63ºC for 4 min, 35 cycles of 94˚C for 1 min (denaturation) , 63˚C for 2 min (annealing), 70˚C for 10 min (final extension) and 25ºC held as the final step. To fractionate the MPCR DNA product eletrophoretically, 10 µl of the MPCR product was mixed with 2 µl 6 × loading buffer on a 2% agarose gel containing 0.5 mg/ml ethidium bromide.

RESULTS

From 58 samples studied, 37.9% were diagnosed histopathologically as CIN I, 36.2% as CIN II, 15.5% as CIN III and 10.30% of the cases were insufficient (Table 1). In relation to HPV types found in the studied pre-malignant samples, 13.6% of CIN I were infected with HPV type 6, 4.5% with the association of types 6 and 11 and 9% with type 11. In these samples, HPV type 16 was not found, in that this was associated with condylomatosis lesions of the genital area, where 69.5% of the samples were negative and 4.3% were insufficient. However, 9% of CIN II was positive for HPV type 16, while 4.5% showed type 6, whereas 81% were negative and 4.5% of the material was in-sufficient for a molecular study. In CIN III, HPV 16 was identified in 15.3% of samples, while 15.3% in HPV 6, whereas 38.4% were negative and 30.7% of the material was insufficient. Consequently, HPV 16 of oncogenic high risk was found in CIN II and III lesions (Table 2).

Ultrastructure

At the ultrastructural level, a sample with a histophatological diagnosis of CIN I, showed reduplicated capillary basement membrane (Figure 1). In CIN II positive for HPV type 16, many mast cells were observed (Figure 2). In CIN III positive for HPV type 16, mast cells were found too, and in the endothelial capillary, neither caveola nor pinocytotic vesicles were observed (Figure 3). In a sample of the invasive cancer, the cervix was infected with HPV type 11 and its oncogenic low risk, and it observed prolongations of the endothelial cytoplasm toward capillary lumen and the enlarged endothelial wall. Also, lysosome, caveola and pinocytotic vesicles of unstable size were shown in Figures 4 and 5.

DISCUSSION

All CIN samples exhibited HPV type 6, considering its oncogenic low risk that is frequently associated with condyloma acuminate and oral warts, which concomitantly occur with genotype 11. In the study's cases, type 16 was detected in samples diagnosed by histopathological techniques in CIN II and III. This viral genotype could be related to angiogenesis (Hong et al., 2005) through stimuli of production and secretion of endothelial growth factors, as in the case of infection by Epstein-Barr virus at the level of lymphoblast cells, where the virus may potentially contribute to viral patho-genesis (Ogata et al., 2003).

Similar observation was performed in the paraneoplastic phenomenon associated to retinoblastoma. In this case, similar capillary alterations were found with proliferation, degeneration and endothelial necrosis in the paraneoplastic phenomenon associated with broncogenic carcinoma (Finol et al., 2001; Tonino et al., 1991). In CIN II, HPV that is positive for type 16 is considered as an oncogenic high risk due to its frequent association with squamous cells carcinoma of anal and genital tracts (Graterol et al., 2006; Kurman, 1994). In the samples, many mastocytes were observed.

They were found, in the connective tissue and the proximity of capillary and nerve fibers, to be apparently playing an important role in angiogenesis, in the regeneration of normal tissue and in the neoplastic processes (Branca et al., 2006). The presence of many mast cells also was observed in the paraneoplasia phenomenon of patients with cervix carcinoma and broncogenic carcinoma (Tonino et al., 1991). Mastocyte activation induces secretion of granules content by a regulated exocytotic process and the synthesis and secretion of cytokines and lipid mediators, contributing to inflammation. These mediators or its derivatives cause different effects on blood vessels observed in immediate hypersensitivity and a late reaction characterized by

Table 2. Types of HVP in lesions premalignant of uterine cervix (CIN).

	Human papillomavirus (HVP) types										
	HVP 16		HVP 11		HVP 6		HVP 6, 11		Neg.	Total	
	Case	%	Case	%	Case	%	Case	%	Case %		
CIN I	-	-	2	9	3	13.6	1	4.5	16	69.5	22
CIN II	2	9	-	-	1	4.5	-	-	18	81	21
CIN III	2	15.3	-	-	2	15.3	-	-	5	38.4	9
Total	4		2		6		1		39	52	

Note: Samples of cervix were obtained in the uterine cervix pathology, Department of the María Teresa Toro Outpatient Clinic in Aragua State, Venezuela.

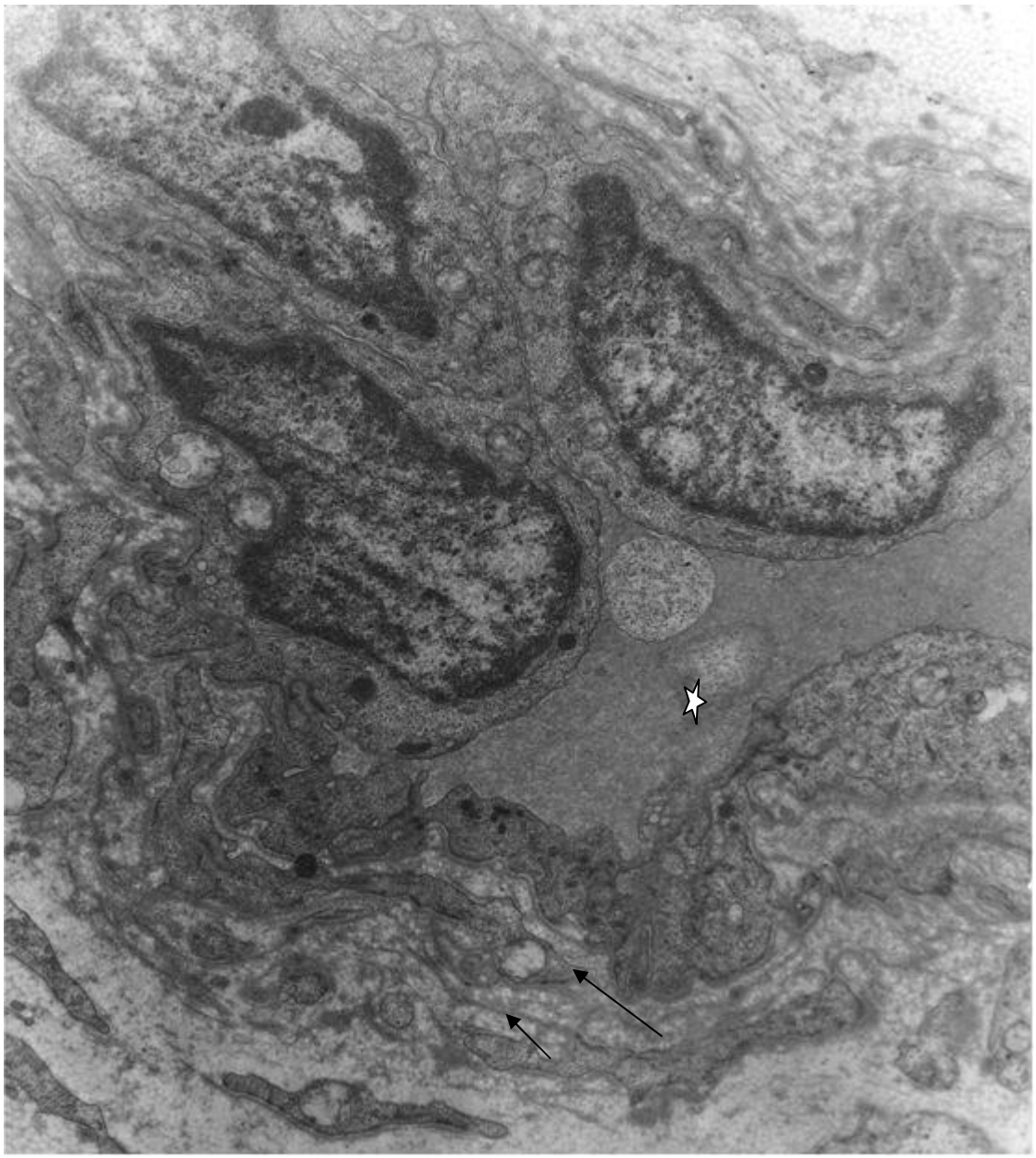

Figure 1. CIN I sample with HVP type 6 infections. This shows a capillary with reduplicated basement membrane (arrows) and the endothelial cell cytoplasm that is widened (star). Magnification: 15,000 ×.

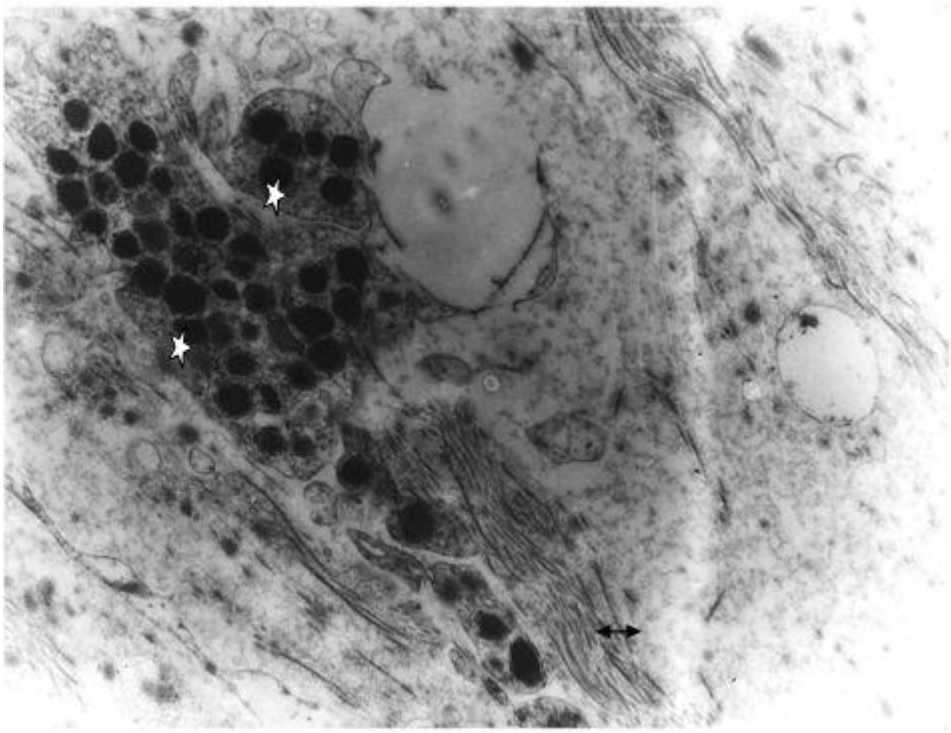

Figure 2. Uterine cervix CIN II sample infected with HVP type 16. Mast cell (stars) is shown. Note: Abundant collagen fibrils: arrows; Magnification: 18,000 ×.

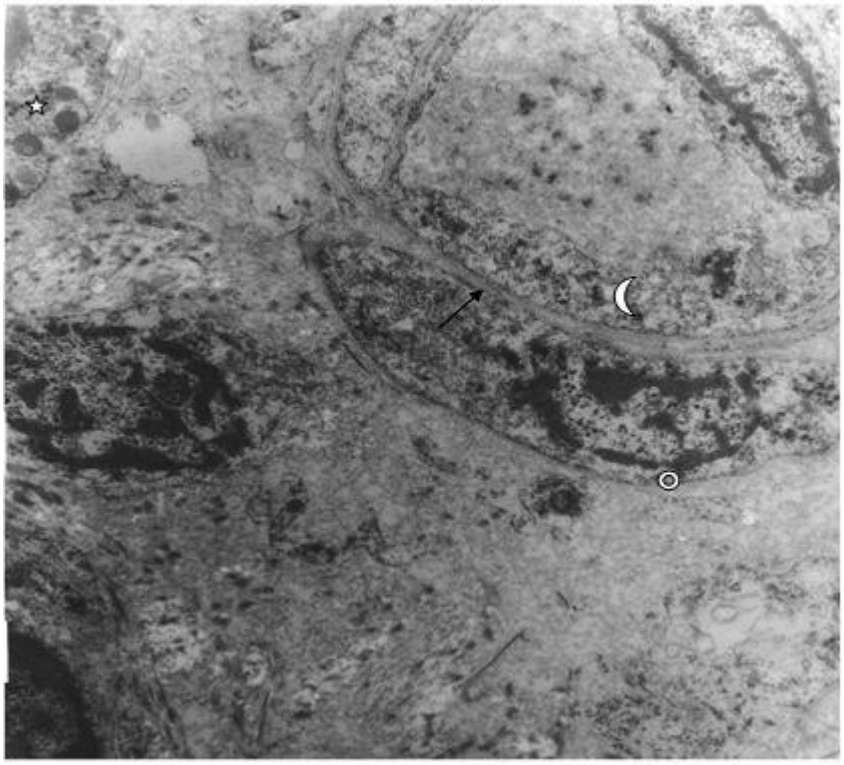

Figure 3. CIN III with infection by HVP type 16. Capillary basement membrane is duplicated (arrows). Endothelial (moon) and pericyte cytoplasms are widened. Note: in the pericote, the pinocytotic vesicles (open circles) are present, while a mast cell (star) is observed. Magnification: 45,000 ×.

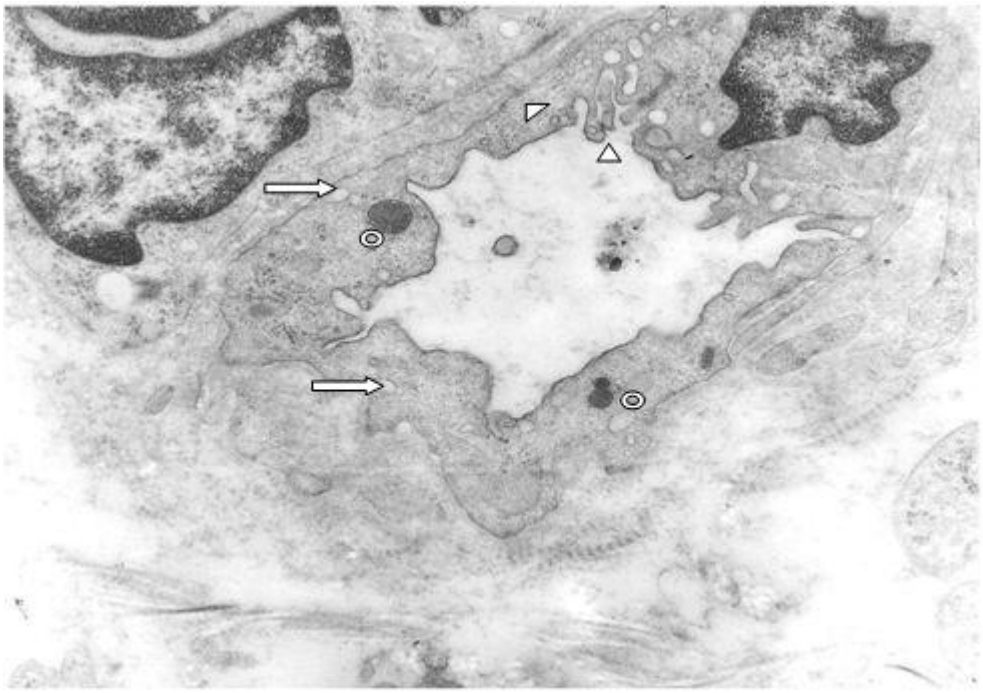

Figure 4. Invasive cervix cancer with HVP type 16. The widened endothelial cell cytoplasm shows prolongations into the lumen (triangles). Note: the primary lysosomes (open circles) and pinocytotic vesicles (arrows) are present. Magnification: 24,000 ×.

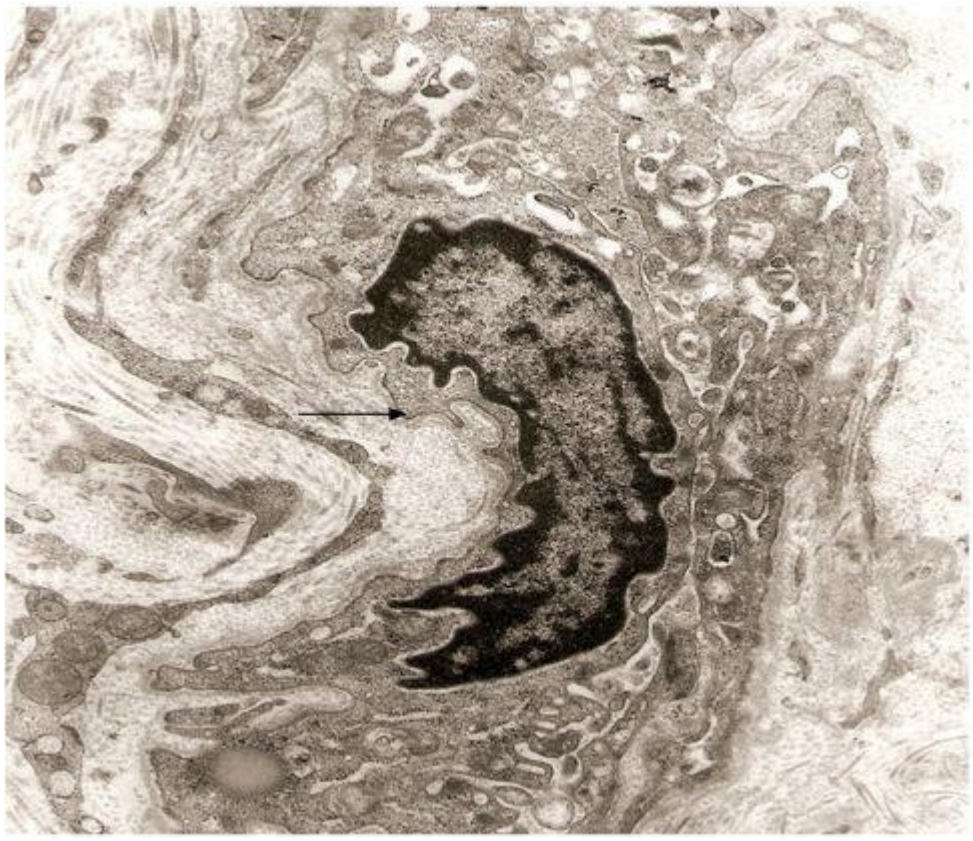

Figure 5. Sample obtained by the cervix uterine of a healthy patient. The basement membrane (arrows) is normal. Magnification: 24,000 ×.

migration of leukocytes and inflammation (Abbas and Lichtman, 2005). This, probably found in the presence of viral genotype of HPV in CIN, may induce mast cells activation at the level of blood vessels and the unloading reaction of biological responses. The inflammatory cells and its regulators may facilitate angiogenesis and promote growth, invasion and metastasis of tumor cells (Lu et al., 2006).

Under the transmission electron microscope, where samples are diag-nosed as CIN III positive for HPV type 16, mast cells were found to invaginate toward capillary lumen and in the endothelial capillary. As a result, neither caveola nor pinocytotic vesicles were observed. Capillary basement membrane reduplication is probably an evidence of vessels alteration that could represent the beginning of the activation processes in angiogenesis. In the transmission, some hormonal signals formed vesicles in association with receptors that are so called caveolas. This represents a special way for the concentration and ingestion of par-ticles and also for the fusion to specific receptors for some molecules. Inversely, these vesicles fuse to plasma membrane in order to pour extracellular matrix specific substances as proteoglicans (Paniagua et al., 2003). In this way, they exteriorize biological products of mast cells and other cells which participate in the reaction for immediate and late defense and chronic inflammatory processes. The role of cells mast in the development and progression of cancer has not been clarified, but it is evident that its numbers in various tumors increase with the progression of lesion. Also, increment of mast cells has been observed in areas of inflammation around malignant cells and a high number of "*in situ*" carcinomas, while other investigators have observed increment of mast cells in the cancer progress (Benítez-Bribiesca et al., 2001; Oner, 2005). It is possible that the changes of the capillary in CIN of the patient with HPV 16 could be related to the viral activity or the activity of the mast cell or to both activities.

In a sample of invasive cancer, the cervix was infected with HPV type 11, which is an oncogenic low risk and it observed prolongations of the endothelial cytoplasm toward capillary lumen and the enlargement of endothelial wall, with lysosomes, caveolae and pinocytotic vesicles of unstable size. This is to demonstrate that the solid tumor capillary may be mixed with endothelial cell and malignant cell. The infiltration of tumor cells in the vascularitation favors tumor growth and metastasis (Lozano 2000; Ross et al., 2005). However, the changes observed in the sample of invasive cancer of the cervix may be highly associated with the vascular endothelial growth factor and the influence of the papillomavirus in the angiogenesis. The enlargement of endothelial cytoplasm may be predictive of the vascular invasion and meta-stasis as in the hepatic tumors (Yao et al., 2005) and the esophageal squ-amous cell carcinoma (Tian et al., 2006). In other samples, plasmocytes with abundant rugose endothelial reticule was observed near the degenerated blood vessel. This was observed in other cancer samples, such as hepatocellular cell infiltrate and the angiogenesis (Ch'nh et al., 2006; Peng et al., 2005). Moreover, an important increase of mast cell was observed in the progression of premalignant and malignant lesion (Benítez-Bribiesca et al., 2001). The virus itself is a high risk oncogenic element, in that it produces alteration of normal cells and induces angiogenesis. HPV produces cellular alterations observed in uterine cervix cancer and other types of cancer. It is pos-sible that the HPV plays two roles in the process of cancer, by producing changes in the structure of infected cells and favoring the growth of tumoral cells by inducing angiogenesis.

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