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Ultrastructural pathology of vascular endothelial in patients with premalignant and malignant cervix lesions infected with papillomavirus

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ABSTRACT

The purpose of this study was to determine the ultrastructural of vascular endothelial and relationship of the human papilloma virus (HPV) infection in patient with cervix lesions. Fifty-eight (58) tissue samples from the uterine cervix were obtained from patients with cervical intraepithelial neoplasia (CIN) and human papillomavirus infection at a public outpatient clinic in Aragua State, Venezuela. For the ultrastructural study, routine transmission electron microscopy techniques were used as well as polymerase chain reaction (PCR) for HPV. In the ultrastructural study (figure 3) NIC I sample with HPV type 6 infection. This section shows a capillary with reduplicated basement membrane and the endothelial cell cytoplasm widened (figure 4) Uterine cervix NIC II sample infected with HPV type 16. A mastocyte is shown. Note abundant collagen fibrils.(figure 5) NIC III sample with infection by HPV type 16. Capillary basement membrane is duplicated . Endothelial and pericyte cytoplasms are widened. Note in the pericyte the presence of pinocytotic vesicle.(figure 6) Invasive cervix cancer with HPV type 16. The widened endothelial cell cytoplasm shows prolongations into the lumen. Note the presence of primary lysosomes and pinocytotic vesivles.HPV11 was identified and both endothelial cytoplasm prolongations into the lumen and thickening of the endothelial wall were clearly seen. Changes of microvascular tissue in premalignant lesions and increased thickness of the endothelial capillary walls in malignant lesions of the uterine cervix indicated the activation of

angiogenesis.

1. Introduction

Tumor angiogenesis is required for the transition from a small harmless group of tumor cells to a large malignant tumor (Lozano 2000). Neovascularization is induced by continual development of blood vessels closely associated with the proliferation, invasion and metastasis of solid tumors (Tsunenari 2003, Ross 2005) and is related to a multiple variety of growth factors produced by neoplastic and inflammatory cells infiltrating tumor tissue. In other instances, invading macrophages and endothelial cells have been observed in malignancies.

The association of angiogenesis with the biological and histological behavior of tumor cells is not yet sufficiently clear. In addition, the debate over tumor vascularity continues, and decisive evidence for determining the histological type of tumors and degree of differentiation of tumor cells still has to be established. The endothelial cell has an important role in blood homeostasis, as its functional properties change in response to diverse stimuli. This process, known as endothelial activation, is also responsible for many types of vascular pathology. Inducers of endothelial activity are bacterial, viral and cytotoxic agents as well as lipidic products and hypoxia (Vidal S. 2002, Ross 2005). Recently attempts have been made to correlate vascular density as a measure of angiogenic response and tumor malignancy for prognostic purposes in mammary, ovarian, prostatic and colonic cancers (Vidal S. 2002, Ross 2005).

Colposcopy, a technique which allows evaluation of vascular tissue in premalignant lesions of uterine cervix, has proven to be a reliable method of obtaining a biopsy for the final diagnosis by histopathological study. The coloposcopic index, based on lesion borders, color, vascular patterns and reaction to iodine, may improve differentiation between CIN I and the more significant CIN II and III (Baillie 1995). The vascular pattern of CIN I (Fig. 1) observed by colposcopy is formed by uniform vessels of a small caliber frequently disposed loosely and randomly in a horizontal network. There may be non-dilated capillary loops present that exhibit a uniform vascular caliber arranged in a vertical path towards the surface. However, CIN II and III have revealed abnormal vasculature (figure 2,3). After applying acetic acid (Aa), most CIN III lesions are seen as white spots that lack any vascular pattern.

This is due to constriction of very narrow vessels produced by the Aa application induced edema. Following this procedure it is possible to observe the classic dots in mosaic that are found in a small percentage of high-risk lesions. Dilation of vertically oriented vessels produces a randomly directed structural disposition. Angiogenic factors generated by high-risk lesions cause the development of prominent dilated conducts which, as seen under colposcopy, separate superficial epithelium into a group of individual blocks or mosaic patterns. These patterns increase in thickness and intercapillary distance that can further develop as lesion malignancy advances (6). The extent of the vascularity in a determined region of a malignant lesion depends on the balance of stimuli and inhibitors in angiogenesis produced by tumor cells (Lorinz 1996, Tsunenari 2003). Recently, proliferative cellular activity has been considered as a marker of potential malignancy in diverse carcinomas, some studies demonstrating a close relationship of tumor angiogenesis and cellular proliferation with clinical results (Tsunenari 2003, Hanahan 1996). Furthermore, rapid tumor vascularization posterior to inhibitory therapy reversals of vascular endothelial growth factor (VEGF) (Maeda 1996) and an association of p53 and VEGF as a predictor of tumor vascularization in bladder cancer (Mancuso 2006) due to a correlation with tumor angiogenesis have been identified. This is important in the formation of new blood vessels, as its expression and consequent microvascular density has an important role in tumor growth and metastasis which suggest their possible value for a prognosis index (Du 2003, Tian 2006). In addition, due to their degranulation, the presence of mast cells that arise from progenitor cells located in bone marrow plays a significant role in the variety of reactions of chronic inflammation and neovascularization (Lozano 2000). In normal conditions, there are no circulating mast cells. Progenitor cells migrate to peripheral tissues as immature cells, differentiating *in situ.* Mature mastocytes are distributed throughout the entire organism, though principally in the proximities of blood vessels, nerves and epithelium. Mast cell activation originates three types of biological response: secretion of granules through a process of regulated exocytosis; synthesis and secretion of lipid mediators; and synthesis and secretion of cytokines. Masts

cells can also secrete tumor necrosis factor (TNF) (Carusos 1997, Abbas 2005). In vascular epithelial cells, this provokes adhesion for leukocytes, neutrophils, monocytes and lymphocytes in inflammatory processes and induces apoptosis of some types of cells. Mast cells have also been related to development and progression of basal cell and squamous cell carcinomas and melanoma. Additionally there is evidence that suggests the intervention of the mast cell in the genesis of malignant cutaneous tumors by its activation of the vascular endothelial growth factor in basal cell carcinoma and melanoma (Ch'nh 2006, Nienartowicz A. 2006). This study proposed to determine the possibility of an ultrastructural relationship of infection by HPV, angiogenesis and cellular infiltrate to premalignant and malignant lesions of the uterine cervix.

2. Materials and methods

Tissue samples obtained from the uterine cervix of 58 patients being seen in the Uterine Cervix Pathology Department of the María Teresa Toro Outpatient Clinic in Aragua State, Venezuela, were studied. At the time of this study, all samples had cytological and histopathological reports of untreated human papilloma virus (HPV) infections. For the ultrastructural study, pieces of cervix were fixed with 3% glutaraldehyde and 1% OsO4, with both fixatives 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 stained with uranyl acetate and lead citrate. Sections were examined with a JEM-1011 transmission electron microscope.

2.1. 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 (50mM Tris- HCL, pH 8.0; 1 mM EDTA and 1% N- laurilsarcosin) containing 0.5 mg/ ml of proteinase- K and incubated for 24 hours at 55ºC. DNA was purified with phenol- chloroform isoamyl alcohol and precipitated by ethanol. The DNA was dissolved in 50 µl of TE buffer.

2.2. PCR assay

HPV status was defined by PCR with the L1 consensus primers MY09 and MY11. This PCR assay used DNA purified from 58 samples of uterine cervix to detect 27 HPV types known to infect the genital tract. To determine specimen adequacy, the GH20/ PC04 human βGlobin target was co-amplified with HPV sequences. Each amplification contained 10 mM Tris- HCl (pH 8.5), 50 mM KCl, 4 mM $MgCl_2$,a 200 µM concentration (each) of dCTP, dGTP, dATP, and dTTP, 7.5 U of AmpliTaq, 2.5 pmol (each) of the Bglobin amplification primers, and 5 to 10 µl (approximately 500 ng) of template DNA. Reactions were amplified in a MJ Research PTC150 thermal cycler by using the following profile: 94ºC for 4 min, 40 cycles of 94 ºC for 1 min (denaturation), 55ºC for 1 min (annealing), 72ºC for 1 min (extension), 72ºC for 5 min (final extension), and 4 ºC hold step. A known positive specimen and a negative (no DNA) specimen were included in each assay as controls.

2.3. PCR assay typing of human papillomaviruses

The HPV typing was carried out using the MPCR Amplification and Detection kit. This kit is 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 2X MPC Buffer Mixture, 5 µl 10X MPCR Primers, 0.5 µl Taq DNA polymerase (5U/µl), 14.5 µl H2O, and 5 to 10 µl of template DNA. Reactions were amplified in a MJ Research PTC150 thermal cycler by using the following profile: 2 cycles 96 ºC for 1 min, 63 ºC for 4 min, 35 cycles 94 ºC for 1 min (denaturation) , 63 ºC for 2 min (annealing), 70 ºC for 10 min (final extension), and 25ºC hold step. To fractionate the MPCR DNA product electrophoretically, 10 µl of the MPCR product was mixed with 2 µl 6X loading buffer on a 2 % agarose gel containing 0.5 mg/ml ethidium bromide.

3. Results

From the 58 samples studied, 37.9 % (22) were diagnosed histopathologically as CIN I, 36.2 % (21) as CIN II, and 15.5 % (9) CIN III. There was insufficient material for analyses in 10.3% (6) of the cases. (Table1). In the HVP types found in the pre-malignant samples studied, 13.6 % of CIN I were infected with HVP 6; 4.5% were associated

with types 6 and 11and; 9% were identified with type 11. In these samples, HPV 16 was not found. CIN II was positive in 9% of HVP 16; 4.5% in type 6; 81% were negative and 4.5% of the material was insufficient for molecular study. In CIN III, HVP 16 was identified in 15.3% of the samples; HVP 6 in 15.3%; 38.4% were negative; and in 30.7% there was insufficient sample material for analyses. High oncogenic risk HPV 16 was found in CIN II and CIN III lesions (Table 2).

3.1. Ultrastructure

At the ultrastructural level, one sample with a histopathological diagnosis of CIN I evidenced duplicated capillary basement membrane (Fig. 1). In CIN II, numerous mast cells were found in HVP 16 (Fig. 2). CIN III positive for HVP 16 also exhibited multiple mast cells, although there were no caveolae or pinocytotic vesicles seen in the endothelial capillary (Fig. 3). In a sample of invasive cancer, the cervix was infected with HVP 11. Although type 11 is oncogenically low risk, prolongations of the endothelial cytoplasm toward the capillary lumen, enlarged endothelial walls, lysosomes, caveolae and pinocytotic vesicles of varying sizes were clearly perceived (Fig. 4).

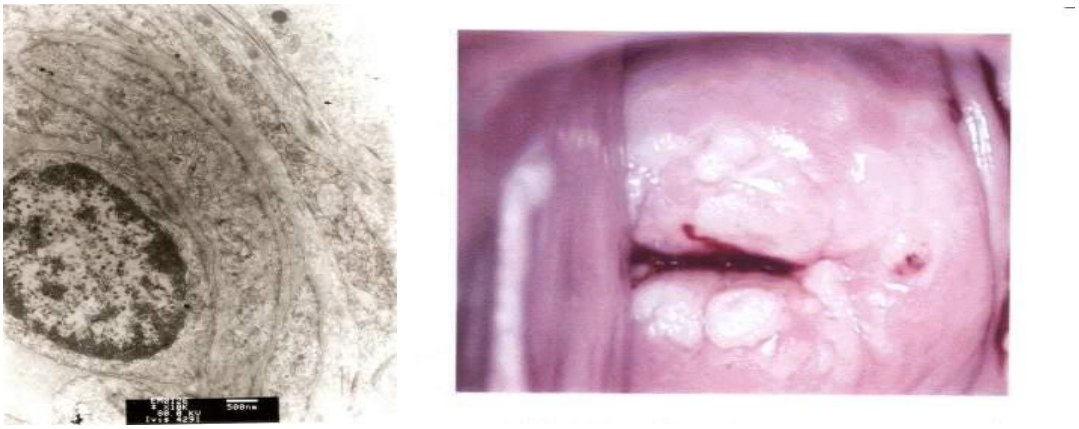

Fig. 1. CIN I TEM, There is a capillary, pinocitica and caveolae, thickened basement membrane vesicles (◄►). Colposcopy (right) allows evaluation of vascular tissue in premalignant lesions of uterine cervix.

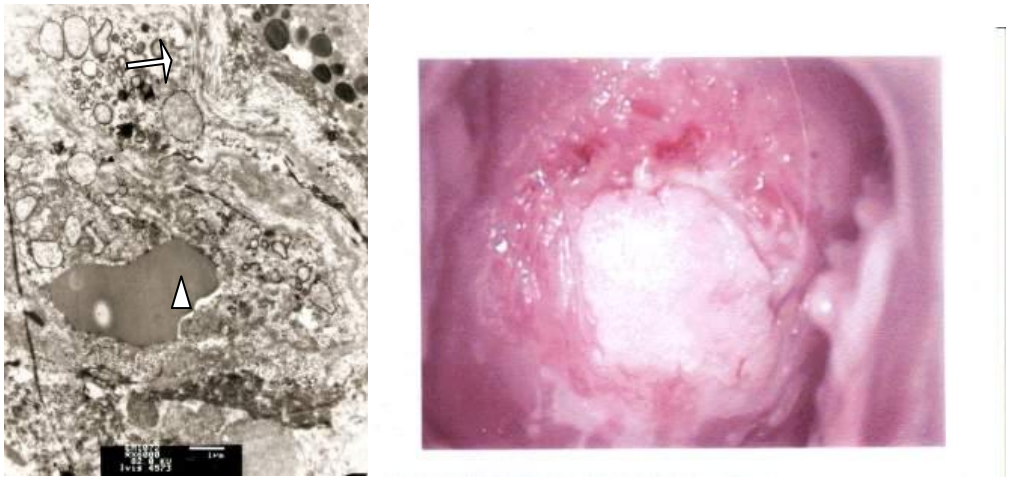

Fig. 2. -CIN II.TEM Shows a mast cell(⇨) and atypical cells ⇦) .Colposcopy (right) allows evaluation of vascular tissue in premalignant lesions of uterine cervix.

4. Discussion

All samples of CIN exhibited the low oncogenic risk HVP 6, which is however, frequently associated with condyloma acuminate, oral warts and concomitantly with genotype 11. The present study detected type 16 HPV diagnosed by histopathological techniques in CIN II and III. This viral genotype could be related to angiogenesis (Hong 2005) through the stimulation of production and secretion of endothelial growth factors, as in the case of the Epstein-Barr virus in lymphoblast cells where it may potentially contribute to viral pathogenesis (Ogata 2003).

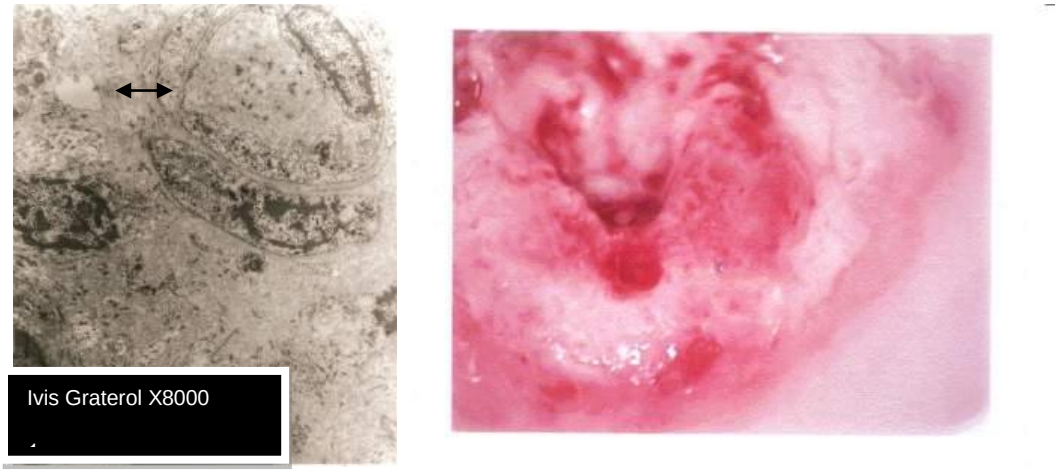

Fig. 3. -CIN III.TEM Altered capillary and degenerate pericytes (◄►) .Colposcopy (right) allows evaluation of vascular tissue in premalignant lesions of uterine cervix.

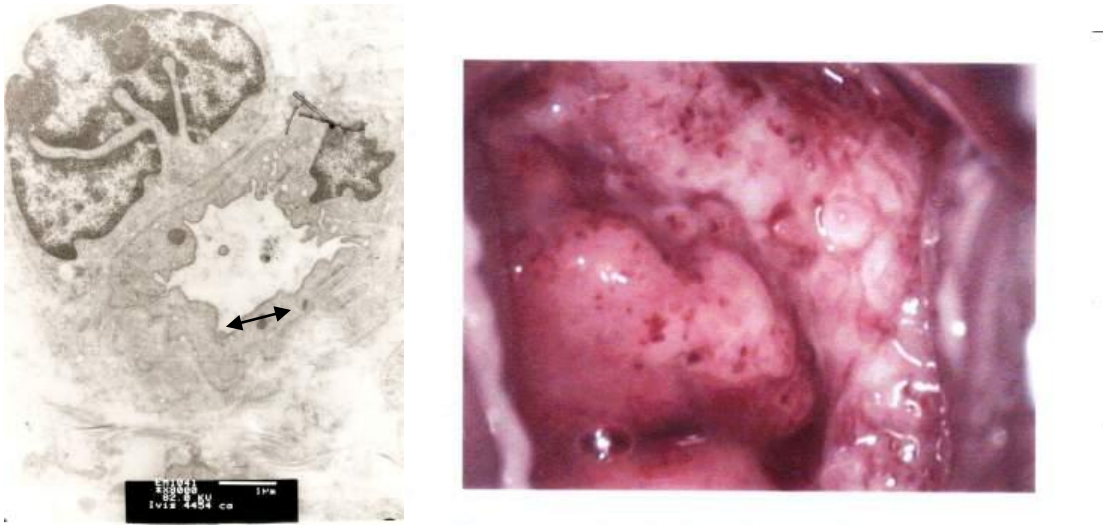

Fig. 4. -TEM Cancer invasive. Capillary with few vesicles, pinociticas and caveolae indicating vascular changes (◄►). Colposcopy (right) allows evaluation of vascular tissue in premalignant lesions of uterine cervix.

A comparable study was carried out in the paraneoplastic phenomenon associated with retinoblastoma. Similarly, capillary alterations with proliferation, degeneration and endothelial necrosis in the paraneoplastic phenomenon were found linked to broncogenic carcinoma (Tonino 1991, Finol 2001). HPV 16 is considered of high oncogenic risk due to its frequent association with squamous cell carcinomas of the anal and genital tracts (Kurman 1994, Graterol 2006). In the present study, numerous mastocytes were detected in the CIN samples. Another study encountered abundant mastocytes in the connective tissue in close proximity to capillary and nerve

fibers, this apparently playing an important role in angiogenesis of normal tissue regeneration and in neoplastic processes (Branca 2006). Many mast cells were also observed in the paraneoplasica phenomenon of patients with uterine cervical and broncogenic carcinomas (Tonino 1991).

Mastocyte activation induces secretion of granule content by means of a regulated exocytotic process, synthesis and secretion of cytokines and lipid mediators, all of which contribute to inflammation. These mediators or their derivatives cause varied effects on blood vessels as has been reported in situations of immediate hypersensitivity and the late reaction characterized by leukocyte migration and consequent inflammation (Abbas 2005). Such effects are probably due to the presence of the viral genotype HVP in CIN that may induce mast cell activation in blood vessels, and as a possible result, the unloading reaction of biological responses may occur. The inflammatory cells and their regulators may facilitate angiogenesis and promote growth, invasion and metastasis of tumor cells (Lu H. 2006).

Under the transmission electron microscope, the CIN III samples with HPV 16 exhibited mast cells invaginated toward the capillary lumen. Neither caveolae nor pinocytotic vesicles were observed in the endothelial capillaries. Capillary basement membrane duplication probably evidences vessel alterations that could represent the beginning of angiogenesis activation. In the transmission of some hormonal signals, vesicles are formed in association with receptors, the so-called caveolae.

This process represents a special mechanism for concentrating and ingesting particles and also for the union of some molecules to specific receptors. In an inverse process, these vesicles fuse to plasma membrane in order to discharge specific substances as proteoglicans (Paniagua 2003) into the extracellular matrix which is a way to exteriorize biological products of mast cells and other cells that participate in the reaction of immediate and late defense and in chronic inflammatory processes. The role of mast cells in the development and advancement of cancer has not been clarified, but it is evident that their number in various tumors increases with progression of the lesions. The increase of mast cells in areas of inflammation around malignant cells in many *in situ*-carcinomas and (in) progressive cancers has been found (Benítez-Bribiesca 2001, Özdemir 2005). It may be that the capillary changes in the CIN patient with HVP 16 could be related to either viral or mast cell activity or to both.

One invasive cancer sample of cervix was infected with low oncogenic risk HPV 11. However, prolongations of endothelial cytoplasm toward the capillary lumen and enlargement of endothelial wall with lysosomes, caveolae and pinocytotic vesicles of various sizes clearly seen perceived.

This demonstrates that solid tumor capillaries may combine with endothelial and malignant cells. Vascularization provides nutrition and oxygen for the infiltration of tumor cells and for the growth and metastasis of tumors (Lozano 2000, Ross 2005).

The changes noted in the samples of invasive cervical cancer with papillomavirus may also be associated with the vascular endothelial growth factor and with angiogenesis. Thickening of endothelial cytoplasm may predict vascular invasion and metastasis as occur in hepatic tumors (Yao 2005) and esophageal squamous cell carcinoma (Tian 2006). In another sample, plasmocytes with abundant rugose endothelial reticule near the degenerated blood vessels were identified. This was also observed in other cancers as hepatocellular cell infiltrate and angiogenesis (Peng 2005, Ch'nh 2006).Other authors found an important increase of mast cells in the progression the premalignant and malignant lesion of the uterine cervix (Benítez-Bribiesca 2001).

5. Conclusion

Changes of microvascular tissue in premalignant lesions and increased thickness of the endothelial capillary walls in malignant lesions of the uterine cervix indicated the activation of angiogenesis.

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