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Angiogenesis Modulators Expression in Culture Cell Lines Positives for HPV-16 Oncoproteins

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Altered angiogenesis response is observed in patients with cervical cancer. In this study we examined whether Human Papilloma Virus (HPV) positive epithelial cells are able to produce angiogenic modulators. When added to human umbilical vein endothelial cells (HUVEC) the media conditioned by HPV-16 positive cells was able to induce proliferation, whereas a contrary effect was observed for media derived from non-tumorigenic keratinocytes. The analyses of angiogenesis modulator's mRNA levels result in a decrease of the antiangiogenic factors TSP-1 and 2 in HPV-16 positive cells. In contrast the expression of the pro-angiogenic molecules: bFGF, IL-8, TGF-β, TNFα, and VEGF were higher in these cells as compared to control keratinocytes. Furthermore the pattern of VEGF isoforms observed in the cells positive for the viral genome point to a preferential induction of the $VEGF_{189}$ isoform. We therefore conclude that cervical cancer cells expressing HPV-16 genome are able to contribute to the pro-angiogenic response that might support tumor growth and invasion of the surrounding tissues.

Key Words: **human papillomavirus Type-16; HPV-16; cervical cancer; angiogenesis; VEGF; TGFβ; TNFα; bFGF; IL-8; TSP-1; TSP-2.**

Cervical cancer is the most common type of gynecological malignancy and the second leading cause of death in women worldwide (1). Recent epidemiological and experimental evidences suggest that almost 99.7% of invasive cervical carcinomas contain and express DNA from Human Papilloma Virus (HPV), being HPV-16 and HPV-18 the subtypes most frequently found (2, 3). The early viral proteins E6 and E7 are continually expressed in HPV-associated cervical cancer tissues and in cell lines derived from cervical tumors (4). The expression of these proteins is known to mediate the oncogenic transformation of squamous epithelial cells. This effect has been correlated with the ability of both E6 and E7 to abrogate the function of the important tumor suppressor genes: p53 and pRb (5, 6, 7). E6 and E7 are known to induce the expression of or to transactivate some transcriptional and growth factors, e.g., *c-fos*, *c-myc*, *c-Jun*, TGFβ (Transforming Growth Factor β) and VEGF (Vascular Endothelial Growth Factor), and these activities have been also correlated to the oncogenic potential of both oncoproteins (8, 9, 10, 11). More recently it has been demonstrated that the expression of E6 and E7 in squamous epithelial cells from the K14HPV16 transgenic mouse is related to the early onset of the angiogenic response and the induction of VEGF expression in tumoral tissues (12, 13). In human cervical cancer, angiogenesis appears since the premalignant stages and it is an important element to characterize disease evolution (14). Increased levels of some direct and indirect inducers of the angiogenic response have been detected in human cervical carcinoma, nevertheless the activity and origin of some of them in the tumoral environment is the subject of present discussion (15). *In vitro* studies directed to identify the source of pro- and anti-angiogenic factors in cervical cancer have analyzed the potential contribution of tumoral cells to the levels of these molecules in patient sera (16, 17). Nevertheless, depending on the conditions used for cell culture, different results has been obtained trying to correlate the expression of some of these angiogenesis modulators with the HPV infection. The present study was undertaken to evaluate whether the expression of HPV-16 oncoproteins in human epithelial cells submitted to serum starvation, could be associated with altered expression of angiogenic modulators. Furthermore, the capacity of the media conditioned by these cells to induce endothelial cells proliferation was tested. Analysis of the results proves that after starvation, the expression of pro-angiogenic molecules is significantly up regulated in HPV-16 positive cells, as compared to HPV-16 negative and nontumorigenic cells.

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TABLE 1

Oligonucleotides Primers and Probes

Gene	Primer	Expected band size (bp)
VEGF	Sense: 5′-CCTGGTGGACATCTTCCAGGAGTACC	479
	Antisense: 5′-CTCACCGCCTCGGCTTGTCA	407
		340
		275
E6	Sense: 5′-ATGCACCAAAAGAGAACTGCAATGTTTCAG	470
	Antisense: 5′-GGATCCTTACAGCTGGGTTTCTCTCTACG	
E7	Sense: 5′-GCAACCAGAGACAACTGATCTCTAC	200
	Antisense: 5′-GGTCCTCCAAAGTACGAATGTCTACG	
GAPDH	Sense: 5′-ATCTCTGCCCCCTCTGCTGAT	450
	Antisense: 5′-AGTGTAGCCCAGGATGCCCTT	

MATERIALS AND METHODS

Materials. The VEGF, bFGF (basic Fibroblast Growth Factor), TSP-1 (Thrombospondin-1), TGFβ, GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase), IL-8 (Interleukin 8), and TNFα (Tumor Necrosis Factor α) cDNA probes used in this study were respectively, gifts of Dr. G. Breier (Bad Nauheim, Germany), Dr. P. Dell'Era (Brescia, Italy), Dr. Noel Bouck (Illinois, USA), Dr. A. Von Gavain (Vienna, Austria), Dr. B. Williams (Cleveland, USA), and Dr. M. Selman (Havana, Cuba). MTT as well as all culture supplements and media were purchased from SIGMA.

Cell culture and preparation of conditioned media. RHEK-1 (18) cells (primary human foreskin epidermal keratinocytes infected with Ad 12-SV40 virus) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 5 μg/ml of hydrocortisone (SIGMA). HaCaT (19) (human epidermic keratinocytes immortalized by multiple passages), CaSki (ATCC: CRL-1550) (HPV-16 positive cervical carcinoma line), HPK-1A (20) (human primary foreskin keratinocytes transfected with the entire HPV-16 genome), and C-33A (ATCC: HTB-31) (HPV negative human cervical carcinoma line) cells were maintained in DMEM supplemented with 10% FBS. When cells reached 95% confluence, the media was depleted of serum (to 0.1%) for 24 h and afterward aspirated from the flask. This conditioned media (CM) was filtered and stored at −80°C until used.

Endothelial cell proliferation. HUVEC (Clonetics, San Diego, CA, passages 8 to 13) were cultured in 2% gelatin-coated flasks, in MCDB131 medium supplemented with 15% FBS, heparin (10 U/ml) and bFGF (0.5 ng/ml). For the growth assay, 100 μl of a 5×10^4 cells/ml suspension of endothelial cells in complete medium were seeded per well of a 96-well culture plate (COSTAR). Media conditioned by epithelial cells was dispensed 48 h later, at sequential dilutions, after an overnight period of starving in MCDB 131 medium with 0.1% FBS. After incubation at 37°C in a 5% CO_2 atmosphere for 72 h, 25 μl of MTT (1 mg/ml) was added to each well, followed 4 h later by the addition of a solubilization-stop solution of acid isobutil alcohol (10% SDS, 0.01N HCl, 50% isobutyl alcohol). The absorbance at 570 nm was determined 1 h later in an ELISA plate reader (Sensident Scan, Merck). All samples were tested in quadruplicate and differences among the controls and the test groups were analyzed using the ANOVA and Bonferroni tests. Results are shown as the percent of proliferation, compared to the control wells maintained in DMEM, supplemented with 0.1% FBS, heparin 10 U/ml. A parallel plate was stained with crystal violet and photographed.

RNA isolation and Northern blot analysis. Cells grown to 95% confluence were serum starved for 24 h. Total RNA was isolated by the Chomczynski single step method (21). After isolation, RNA was fractionated in a 1% agarose-2.2 M formaldehyde denaturing gel and transferred to ZetaProbe GT (BIORAD) nylon membranes. Fixed membranes were hybridized with the different angiogenic modulators cDNA, labeled to high specific activity ($>1 \times 10^9$ cpm/μg) with P^{32}dATP in a random primer extension reaction (22). To normalize amounts of RNA, filters were washed and rehybridized with labeled GAPDH cDNA. Autoradiograms were analyzed by densitometry (GS-700 Imaging Densitometer, BIORAD) using the appropriate software (Molecular Analyst, BIORAD). Results are expressed as relative amounts of specific mRNA to GAPDH levels. Fold induction was calculated by using the adjusted value for RHEK-1 as the basal level except in the case of TSPs were the C-33A value was used for this purpose.

Reverse transcription-polymerase chain reaction and Southern blot. One microgram of total RNA was used for reverse transcription using a RT-PCR core kit (Perkin-Elmer Cetus Corp). cDNA was amplified by PCR in a 50-μl volume reaction using 10 pmoles of each sense and antisense primers (Table 1). With VEGF primer, the 121, 145, 165, and 189 amino acid isoforms were given bands of 275, 340, 407, and 479 bp, respectively. Amplifications were carried out in a thermal cycler (M.J. Research, Inc.) using thermostable DNA polymerase, 1.25 mM $MgCl_2$, pH 9.0, and dNTP at a final concentration of 0.2 mM. For amplification of E6, E7, and GAPDH genes, a $MgCl_2$ concentration of 1 mM was used. For VEGF an initial denaturation step at 95°C for 3 min was followed by 35 PCR cycles (95°C for 30 s, 50°C for 30 s and 72°C for 90 s). This was followed by a final extension at 72°C for 6 min. Cycling conditions for E6, E7, and GAPDH were 35 cycles at 95 for 1 min, 50 for 1 min, and 72°C for 1.5 min. PCR products were analyzed on 2% agarose gels and visualized by ethidium bromide staining on an ultraviolet transilluminator. A Southern blot was performed, using a $VEGF_{121}$ cDNA labeled as previously described (23). The relative amount of each isoform was analyzed by densitometry with the Molecular Analyst Software (BIORAD). The size of the expected bands for all cDNA molecules is shown in Table 1.

RESULTS

Endothelial Cells Proliferation

We examined the effect of the CM on HUVEC proliferation in the absence of exogenous mitogens. 1:4 dilution of CM was selected from initial experiments (not shown), and comparisons were carried out for 1:4 dilutions. The results were expressed as percent of proliferation respect to control cells maintained with an equivalent dilution of DMEM. A significant increase in HUVEC proliferation (among 25 and 50%, $P < 0.001$) was detected in cells treated with the CM derived from tumor cells, while CM from HaCaT cells led

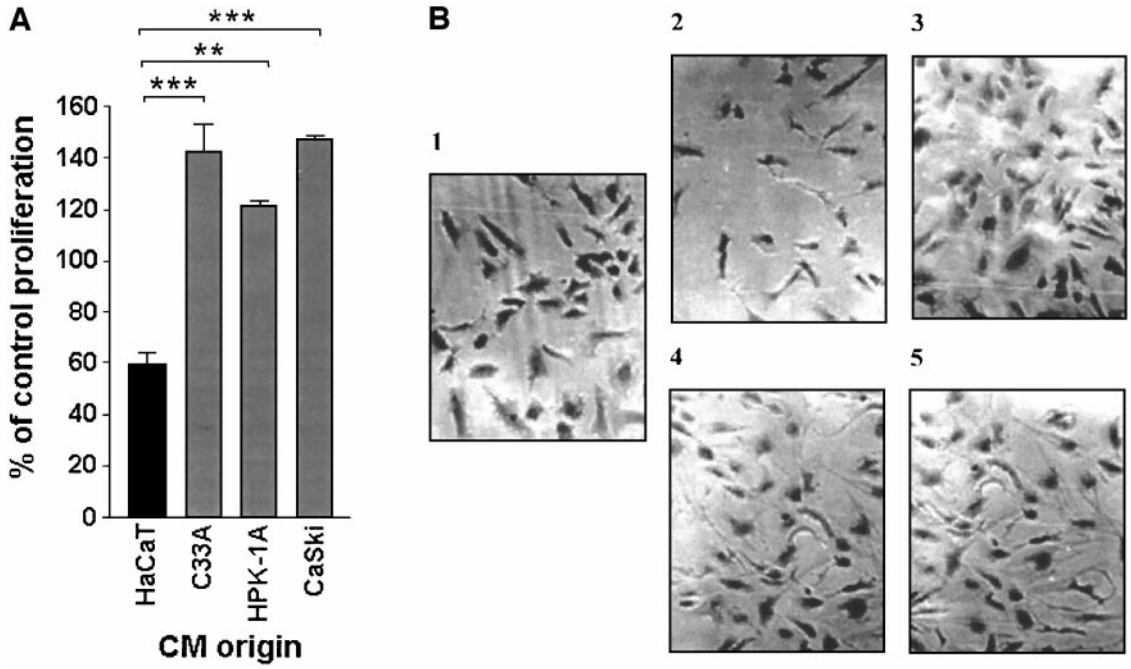

FIG. 1. Mitogenic activity of HUVEC after the addition of culture supernatants from epithelial cells that express or not HPV-16 oncoproteins. (A) The graph shows the percent of proliferation as compared to controls (DMEM, 0.1% FBS) for each conditioned media (CM) as measured through MTT assay. CMs were cleared by centrifugation and filtration, and dilutions were used in a 72-h proliferation assay with HUVEC. The columns and bars represent mean ± SEM of samples performed in quadruplicate. The data illustrated are representative of at least three independently performed experiments. (*** $P < 0.001$, ** $P < 0.01$) (B) Pictures taken from parallel plates stained with crystal violet illustrate the effect observed on HUVEC proliferation and morphology after the administration of the CMs: 1(control), 2(HaCaT), 3(C33A), 4(HPK-1A), 5(CaSki).

to a decrease of about 40% in HUVEC proliferation (Figs. 1A and 1B).

Expression of Angiogenic Modulator's mRNA

In the cases of TNFα, TGFβ, IL-8 (Fig. 2A) and bFGF (Fig. 2B), the mRNA values for HPV-positive cells were among 3.8 to 8 times higher than those found for RHEK-1 cells, while C-33A showed only a twofold increment. When TSP-1 and TSP-2 mRNA levels were analyzed we found a reduce expression of these mRNAs in C-33A cells, as expected. Low levels of TSP-1 mRNA, as compared to those found in RHEK-1 cells, were observed in HPV-positive cells (Fig. 3). We have found lower levels of TSP-2 as compared to TSP-1, but in the case of the former the mRNA levels on HPV positive cells drop 4 times while TSP-1 mRNA was only two times higher in non-tumorigenic RHEK-1 cells.

A two- to threefold increment in VEGF transcription was observed in cell lines derived from cervical cancer, positive for the viral genome, as compared to tumoral cells not bearing HPV-16 sequences (C-33A), and a fivefold increase with respect to immortalized but non-tumorigenic keratinocytes (RHEK-1) (Fig. 2B). Even lower levels of IL-8, TGFβ, TNFα, bFGF and VEGF mRNA were detected for HaCaT cells (not shown).

Analysis of HPV Oncoproteins and VEGF Isoforms Expression

The expression of HPV-16 E6 and E7 oncoproteins was detected in the cells known to be positive for the viral genome: CaSki and HPK-1A, whereas C-33A, HaCaT, and RHEK-1 cells resulted negative for the tested transcripts (Fig. 4). RT-PCRs using VEGF internal primers revealed the expression of four of the isoforms already described for VEGF (121, 145, 165, and 189), that were observed in the ethidium bromide stained gel (Fig. 5A). A subsequent Southern blot hybridization confirmed the nature of the four bands detected, and also revealed the existence of an additional band with a size of approximately 680bp (Fig. 5B). A comparison among the cell lines and the expression of the VEGF isoforms was carried out taking as basal levels those found in HaCaT cells. The results are expressed as fold induction, with respect to basal levels. Enhanced expression of all of the mRNAs isoforms was detected in tumor-derived cell lines as compared to HaCaT. In the case of the $VEGF_{121}$, $VEGF_{145}$, and $VEGF_{165}$ isoforms the transcripts levels were between 1.6 and 2.3 times higher for HPV-16 oncoproteins positive cells. For the $VEGF_{189}$ isoform an increase in 30 times was found in HPV bearing cells as compared to HaCaT levels. The band observed at 680bp for CaSki and HPK-1A cells

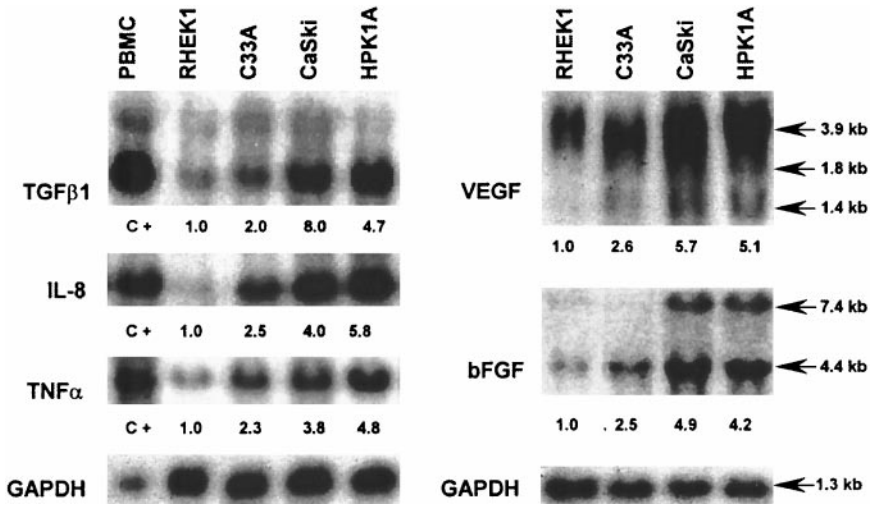

FIG. 2. mRNA expression of proangiogenic molecules. Epithelial cells were submitted to serum starvation for 24 h. Total RNA was isolated and analyzed by Northern blot hybridization with the indicated probes. (A) Expression of the indirect inducers of angiogenesis TGFβ, IL-8, and TNFα. Peripheral blood mononuclear cells were used as positive controls. (B) Expression of the direct angiogenic factors VEGF and bFGF. Images from the autoradiograms were densitometrically scanned, and values were normalized to GAPDH. Numbers below each sample represent fold increase signal versus RHEK-1 cell line levels.

was not detected for HaCaT cells. In the case of C-33A cells the signal corresponding to this 680bp band was 3 times lower than those observed for HPV-16 positive cells (Fig. 4C).

DISCUSSION

The arising of new blood vessels is a characteristic of a number of pathological and physiological processes. Equilibrium among activators and inhibitors of the neovascularization tightly control the event in most tissues (24). When there is no such tight control as in pathological situations (i.e., arthritis, psoriasis, and cancer) blood vessels start to proliferate and aberrant capillary networks are created. In the case of cancer is precisely the switch to an angiogenic phenotype what promotes the growth of the tumor beyond 2 mm^3 and sustain the further metastatic process (25, 26).

Here, we examined the mechanism underlying the upregulated angiogenesis that accompanies the cervical cancer lesions. We have shown that tumoral cells secrete to the CM an activity or activities, that stimulate endothelial cells proliferation. A similar result has been obtained by Rak *et al.* for cells derived from other tumor types or transformed with oncogenes such as ras and raf (27).

Through time many inducers of the angiogenic processes has been identified and among them many have also been related to cervical cancer. For example a study of cytokines and growth factors in the sera of women in various stages of cervical cancer indicated that increase levels of IL-2, IL-6, IL-7, IL-8, bFGF, TNFα, TGFβ, TNFβ, and GMCSF, all of them related to an immune response and potentially angiogenic, are

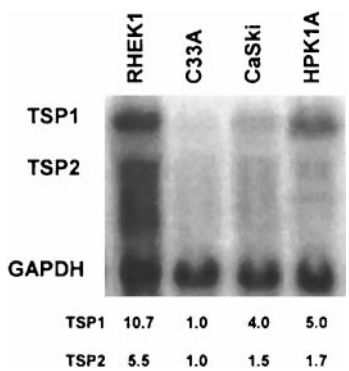

FIG. 3. mRNA expression of TSP-1 and TSP-2. Total RNA isolated from 24 h starved epithelial cells was analyzed by Northern blot hybridization. A 4.4-kb fragment of the pcDNATSP plasmid corresponding to TSP-1 sequence that also binds to homologous regions of the TSP-2 sequence was used as probe. GAPDH probe was also included in the hybridization mixture. Images from the autoradiograms were densitometrically scanned, and values were normalized to GAPDH. Numbers below each sample represent fold increase signal versus C33A cell line levels.

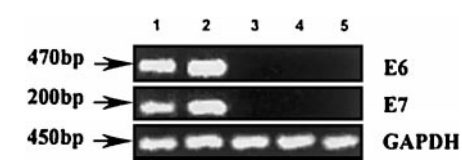

FIG. 4. Detection of HPV-16 oncoproteins transcripts by RT-PCR. HPV-16 E6, and E7 oncogenes were amplified from different cell lines. Lane 1: HPK-1A, lane 2: CaSki, lane 3: HaCaT, lane 4: RHEK-1, and lane 5: C33A. The transcript size is shown at the right side.

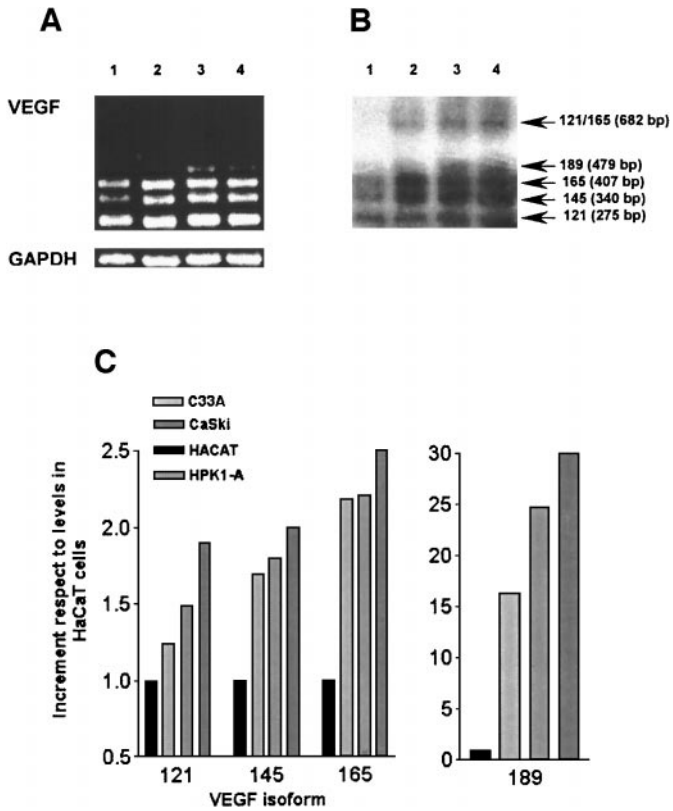

FIG. 5. Expression of VEGF isoforms in epithelial cells. The PCR products were amplified from cDNA templates prepared from the same RNAs used in Northern blot assays, and were analyzed by ethidium bromide staining (A), and Southern Blotting with $dATP^{32}$ end-labeled $VEGF_{121}$ cDNA (B). Lane 1, HaCaT; lane 2, C33A; lane 3, CaSki; lane 4, HPK-1A. The arrows at the left side indicate the isoforms observed and their size, that were determined relative to 50kb DNA ladder markers (Gibco BRL). Autorradiographic signals were evaluated and the results were normalized to the GAPDH signal. Fold induction respect to HaCaT values is shown in graphic (C).

associated to the progression of the disease (15). The investigations related to the origin of these proteins seem to agree in the fact that cervical cancer cells could be the source of TGFβ and TNFα (28, 29). Under certain types of stimulation (i.e., TNFα and IFNγ) Fichorova *et al.* detected an increase in the amounts of IL6, IL-8, IL-7, MSCF, RANTES, and TGFβ in the supernatant of HPV immortalized cells (17). Even so, Woodworth *et al.* has reported a reduction in cytokine mRNA expression after HPV immortalization in subconfluent epithelial cells *in vitro* (16). Nevertheless, the *in vitro* results obtained using fully supplemented subconfluent cultures rarely match with the data obtained *in vivo* or in tridimensional systems characterized by cell–cell contact, glucose starvation and hypoxia (15, 16, 17, 30). In the present study we have addressed whether 24 h serum starvation can modify the levels of angiogenesis related molecules transcripts in confluent cultures. Northern blot analysis results showed that while some cytokines and growth factor mRNA levels remain similar under normal conditions for both control and HPV positive cell lines (not shown), once serum was depleted from the culture media significant differences arose among the cell lines tested.

We found increase levels of TGFβ and TNFα mRNA in HPV positive cells as compared to the nontumorigenic control cells. The loss of sensitivity to the negative effects of TGFβ on epithelial cell growth has been related to the development of cervical carcinoma (31), but is not a prerequisite for it. The treatment of monolayer cultures of HPV positive cells with TGFβ inhibit their proliferation, nevertheless this effect is totally different in organotypic cultures, were TGFβ is able to promote cell differentiation and a dose dependent increment in HPV-16 E7 mRNA (28). A similar behavior has been described for the activity of TGFβ on the angiogenic process since the cytokine inhibit endothelial cell proliferation *in vitro* but induces angiogenesis *in vivo* (15). The same behaviour was described for TNFα, which cooperates, with TGFβ in the recruitment of cells involved in the inflammatory events, frequently generated in tumor surroundings, and indirectly activates the angiogenic event. TNFα has also shown inhibitory activity on epithelial cell proliferation, but in the case of HPV-16 harboring keratinocytes a resistance to this effect and increased cell tumorigenicity were associated with the decreased expression of TNFα receptor (29).

Another mediator of inflammation, recently described as a potent inducer of the angiogenic process, is IL-8. This molecule is able to directly induce proliferation and motility by interacting with the IL-8-type I receptor in endothelial cells (32). The increment of this chemokine in the sera of cervical cancer patients has been linked to tumor progression (15). Our results reveal that HPV-16 positive cells submitted to stress conditions could be a source for IL-8. Similar results were obtained for bFGF, an angiogenic factor that is expressed associated to the increase malignancy of many tumor types including cervical cancer, where its activity is frequently described linked to the induction of collagenase and uPA (urokinase-like Plasminogen Activator) on endothelial cells (33, 34).

Angiogenesis control has also been related to the expression of some inhibitors of the process. Among the most interesting ones is the family of thrombospondins (TSP) and their fragments. One of the main regulators of the transcription of TSP-1 is the tumor suppressor gene p53 (35). The transcriptional activity of p53 is repressed by HPV-16 E7 oncoprotein and the protein is also targeted to degradation by HPV-16 E6 (6, 7). Accordingly, we found lower levels of TSP-1 transcript in HPV positive cells as compared to controls, and a strongly reduced expression on C-33A cells, where p53 is not functional. Low expression of TSP-1 has been directly related to malignant progression in melanoma,

lung, and breast carcinoma cell lines, since the early expression of this molecule in the tumor could inhibit tumor-induced angiogenesis (36). A similar situation was described by Nickoloff *et al.* in psoriatic keratinocytes, where low expression of TSP-1 cooperates with an increase in IL-8 expression to induce endothelial cell proliferation (24). As described by Dameron *et al.*, we observed lower levels of TSP-2 as compared to TSP-1 (35). Nevertheless, the levels of TSP-2 transcript were more affected by HPV16 oncoproteins expression, than the levels of TSP-1. Interestingly, recent reports suggest that TSP-2 is even more anti-angiogenic than TSP-1 (37) and studies in colon cancer patients show a correlation among decrease TSP-2 expression and the metastatic potential of the original tumors also correlated to the increment of $VEGF_{189}$ (38).

In cervical cancer some authors have described an increase on VEGF expression associated to the expression of viral oncoproteins E6 and E7 (12, 13). We have also observed a significant and differential increment on VEGF transcription in cells expressing the viral oncoproteins. Some of the factors already discussed could be implicated in this induction, as many are capable of activate the transcription of VEGF gene via the promoter (i.e., bFGF, TNFα, and TGFβ) (39). It is also known that p53 exert a negative regulation on this promoter, but recent results discard this possibility, as the induction of VEGF transcription by the viral oncoprotein E6 remains equal in p53−/− fibroblasts (11). Since IL-10 has a marked effect on regulating the production and secretion not only of VEGF but also of other cytokines with angiogenic potential (40), we addressed the matter of the correlation between HPV-16 oncoproteins expression and this cytokine. Even though high levels of IL-10 have been found in the sera of cervical cancer patients (15) (stages II, III, IV), we didn't found evidences of IL-10 mRNA expression in any of the epithelial cell lines tested.

Lately, most of the interest has been displaced to the kind of isoform of VEGF that prevails in tumors (41, 42), since in nearly all cases studied, the up regulation of the gene has been widely described. Our results indicated that even when all of the isoforms were over expressed in cervical cancer cell lines as compared to non-tumorigenic keratinocytes, isoform 189 is selectively up regulated in the cells expressing HPV-16 oncoproteins.

Most of the studies related to the isoforms point to $VEGF_{121}$ and $VEGF_{165}$ as the isoforms highly expresses in tumor derived cells, and this fact has been correlated to the aggressiveness and metastatic potential (41, 42). These isoforms are not retained by the extracellular matrix as occurs with $VEGF_{189}$ and $VEGF_{206}$, and in the case of $VEGF_{165}$, this isoform is able to interact not only with VEGFR1 and VEGFR2 but also with the co-receptors neurophilins, that account for the activation of the endothelial cells. Nevertheless, $VEGF_{189}$ appears to be related to the malignant progression of colon lesions. Recent investigations on the molecular species generated from $VEGF_{189}$ confirm the biological actions expected for an angiogenic/survival factor such as VEGF: The molecule is non mitogenic when remains bound to extra cellular matrix, but turns into a strong mitogen with weak ability to circulate when is processed by urokinase or uPA ($VEGF_{110}$) (43). High levels of uPA has been reported for cervical cancer (44), so a loop might be established in HPV positive cells that contribute to the generation of an endothelial cell mitogen ($VEGF_{110}$) as potent as $VEGF_{165}$, with local action. Nevertheless, further studies are needed in order to test whether VEGF isoforms are differentially expressed or not in cervical cancer patients, and if this fact is related to the stage or vascularization of the lesion.

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