SPANDIDOS PUBLICATIONS

The pro-angiogenic factors stimulated by human papillomavirus type 16 E6 and E7 protein in C33A and human fibroblasts

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Received July 26, 2008; Accepted September 15, 2008

DOI: 10.3892/or_00000185

Abstract. To investigate the pro-angiogenic factors stimulated by human papillomavirus type 16 E6 and E7 protein in C33A and human fibroblasts. HPV-16 E6 and E7 genes were transfected into C33A and HFB to detect the profiling of angiogenesis-associated factors with the TranSignal angiogenesis antibody array. The mRNA and protein levels of the cytokines were examined by traditional RT-PCR and Western blotting in both cell lines before and after transfection of HPV-16 E6 and E7. HPV-16 E6 and E7 genes were successfully transfected into C33A and HFB cells. On the sheet of antibody array, after transfection of HPV-16 E6 and E7, 6 other cytokines, Ang-1, FGFα, HGF, IL-6, IP-10 and PIGF besides VEGF, were detected at higher levels in C33A cells. Expression of 7 other cytokines besides IL-8, Ang-1, IL-1α, IL-1ß, HGF, IL-6, VEGF and PIGF increased in HFB cells. The common cytokines in both cell lines were Ang-1, HGF, PIGF and VEGF. The mRNA and protein levels of the four cytokines were verified to increase by traditional RT-PCR and Western blotting in both cell lines after transfection of HPV-16 E6 and E7. Multiple pro-angiogenic cytokines could be stimulated by HPV-16 E6 and E7 protein both in cervical cancer cell line and normal human diploid cells. Anti-angiogenesis therapy may be effective alone or in combination with biologic means aimed at E6 and E7 in the treatment of cervical cancer.

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Abbreviations: HPV, human papillomavirus; HFB, human fibroblast; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; PlGF, placenta growth factor; Ang-1, angiopoietin-1; FGF, fibroblast growth factor

Key words: human papillomavirus, angiogenesis, vascular endothelial growth factor, angiopoietin-1, hepatocyte growth factor, fibroblast growth factor

Introduction

Cervical cancer remains a major worldwide health problem, especially in developing countries. Over the last few decades great advancement has been made in determining the molecular genetics of the development of cancer. The role of human papillomavirus (HPV) infection in the development of cervical cancer is a major player in the genetic abnormalities described thus far. Persistent infection with genital high-risk HPV types, e.g. HPV-16 or HPV-18, contributes to the development of genital cancers, which are characterized by deregulated, high-level expression of the HPV early genes E6 and E7. Previous studies have demonstrated that HPV-16 E6 and E7 protein may disturb the normal procedure of cell life cycle, to inhibit apoptosis, to promote G1/S and G2/M phase spanning, and to activate telomerase (1-4). Altered angiogenesis response is observed in patients with cervical cancer and the HPV oncoprotein E6 may contribute to tumor angiogenesis by direct stimulation of the mediators of angiogenesis such as VEGF gene (5-10). Angiogenesis begins early in the progression of cervical disease from mild to severe dysplasia and on to invasive cancer (11).

Expansion of malignant tissues and tumor metastasis are dependent on neovascularization, which is accomplished by angiogenesis, vasculogenesis, and vascular remodeling. Angiogenesis is normally kept in check by the presence of activator and inhibitor molecules. Activators stimulate repair and growth of blood vessels while inhibitors signal these processes to stop (12-14). To induce angiogenesis, the balance is shifted in favor of blood vessel formation by increasing the amount of activators and/or reducing the number of inhibitors. Almost two dozen proteins, both activators and inhibitors, have been identified that affect angiogenesis. The unusual features of tumor blood vessels represent the consequence of an imbalanced production of various angiogenic factors and the hypoxic environment within the tumor tissue (15-17).

Table 1. The parameters of the indicated genes in PCR, followed with each reaction procedure.

Factors	Primer	Degenaration	Annealing	Extension	Reaction cycles	Size of products
		Temperature time (sec)	Temperature time (sec)	Temperature time (sec)		
ß-actin						
Forward	5'-AGC CAT GTA CGT TGC TAT CC-3'	95˚C (30)	58˚C (45)	72˚C (45)	25	498 bp
Reverse	5'-TTG GCG TAC AGG TCT TTG C-3'					
HGF						
Forward	5'-GTG ACA TTC CTC AGT GTT CAG-3'	95˚C (30)	56˚C (40)	72˚C (45)	33	800 bp
Reverse	5'-GTG GTA TCA CCT TCA CAA CG-3'					
Ang-1						
Forward	5'-CTA CCA CCA ACA ACA GTG-3'	95˚C (30)	50˚C (30)	72˚C (45)	34	497 bp
Reverse	5'-GTA GTC GAA CAC TAC ACT G-3'					
PGF						
Forward	5'-AAA GGA GGA GAC CCA AGG-3'	95˚C (30)	54˚C (30)	72˚C (45)	34	596 bp
Reverse	5'-TCC AGA GCT GAA TGT TCT G-3'					
VEGF						
Forward	5'-CAG CTA CTG CCA TCC AAT CGA-3'	95˚C (30)	52˚C (30)	72˚C (30)	30	200 bp
Reverse	5'-TGC TGG CCT TGG TGA GGT-3'					

To gain a clear picture of the angiogenesis-stimulating pathways affected by cancer cells, it is necessary to investigate the levels of angiogenesis activators and inhibitors in tumors. As the use of microarray technology in genotyping and gene expression experiments has developed over the past decade, nucleic acid arrays are widely produced and utilized in numerous core facilities and research laboratories (18,19). Traditionally, mRNA levels were used to infer the protein content of a cell, but it is now known that this information does not always correlate well (20,21). Protein microarray technology is not as developed as nucleic acid microarray technology, but the potential uses of protein microarrays are equal to, if not greater than, those of the nucleic acid microarray (22-25). The TranSignal™ angiogenesis antibody array we used in the present study is based on the sandwich ELISA method for detecting 19 different angiogenesis-specific cytokines including angiogenesis activators and inhibitors.

In order to know the angiogenic effects of HPV on tumor neovascularization, in the present study we transfected HPV-16 E6 and E7 genes into a human cervical cancer cell line (C33A) and a normal human diploid cells (the human fibroblast, HFB) to detect the profiling of angiogenesis-associated factors using the TranSignal angiogenesis antibody array. Further we examined the mRNA and protein levels of the cytokines by traditional RT-PCR and Western blotting in both cell lines before and after transfection of HPV-16 E6 and E7.

Materials and methods

Cell lines and primary cultured human fibroblast cells. Human cervical carcinoma cell lines, CasKi and C33A were obtained from the American Type Cell Collection (ATCC). CasKi has high copies of HPV-16 genome, and C33A is HPV negative. HFB was obtained in anchoring human corneal tissue and cultured in stable condition. Cellular morphologies were observed by inverted phase contrast microscopy and electron microscope and the proliferation characteristic were described by growth curve and flow cytometry. All the cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2.

Immunocytochemistry to identify human fibroblast cells. The primary cultured human fibroblast cells were identified by immunocytochemistry of vimentin, collagenase type I and keratin and chromosome analysis. The following antibodies were used as below, mouse anti-human vimentin (Santa Cruz, CA), mouse anti-human collagenase type I and keratin (NeoMarker, Fremont, CA). Immunocytochemical analysis was carried out by using the avidin-biotin immuno-peroxidase method.

Plasmid construction and cell transfection. Plasmid p16HHMo containing HPV-16 E6 and E7 genes was a gift from Dr Peter N. Jensen in Washington University, USA. HPV-16 E6 and E7 genes were cleaved by *Bam*HI and *Hin*dIII (Takara Bio Company, Dalian) and cloned into an eukaryotic expression vector pEGFP (Clontech, CA). Recombinant plasmid pEGFP-HPV16E6E7 was transfected respectively into C33A cells and HFB cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. The transfected cell lines were stably cultured using G418 selecting medium. The transfection efficiency was evaluated by inverted fluorescence microscopy.

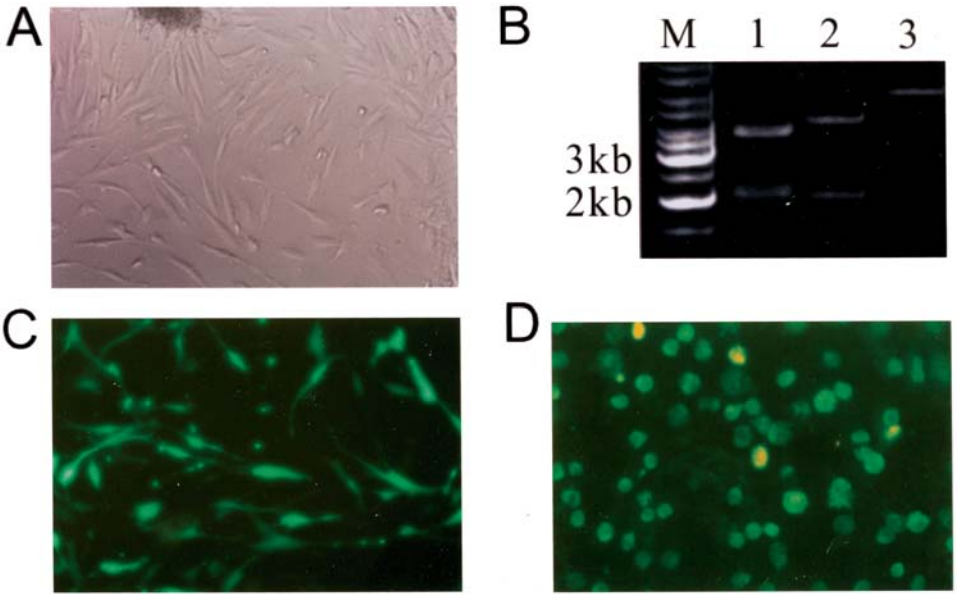

Figure 1. Transfection of HPV16E6E7 into human fibroblast (HFB) cells and C33A cells. (A) Primary cultured human fibroblast cells showed typical shape of fibrous cells after sticking to the inoculating plate. (B) The electrophoresis analysis of plasmid p16HHMO and EGFP HPV16E6E7. Lane 1, plasmid p16HHMO cleaved by *Bam*HI and *Hin*dIII. The 2095-bp band stands for HPV16E6E7. Lane 2, plasmid EGFP HPV16E6E7 cleaved by *Bam*HI and *Hin*dIII. Lane 3, plasmid EGFP HPV16E6E7 cleaved by *Bam*H. (C) After transfection the green fluorescence inside HFB-E6E7 cells was determined by inverted fluorescence microscopy. (D) The green fluorescence inside C33A-E6E7 cells was also observed.

HPV-16 E6 and E7 protein positive and negative cell models were named as C33A cell, C33A-E6E7 cell, HFB and HFB-E6E7 cell.

Detection of HPV-16 E6 and E7 protein by Western blot analysis. The expression of HPV-16 E6 and E7 protein in the transfected cell lines was detected by Western blot analysis. Equal amounts of cell extracts (150 μg) were electrophoresed on 10% sodium dodecyl sulphate-polyacrylamide gel (SDS PAGE) followed by transferring to polyvinylidene difluoride membranes. The membranes were blocked for 2 h with 5% nonfat milk at room temperature and incubated with corresponding antibodies overnight at 4˚C. The antibodies used in this part were rabbit polyclonal antibody of HPV-16 E6 (Santa Cruz, CA) and mouse monoclonal antibody of HPV-16 E7 (NeoMarker, Fremont, CA). After washing in PBS the membranes were incubated with secondary antibodies at dilution of 1:1000 respectively. Immunoreaction bands were visualized by Hyperfilm ECL imaging system (Amersham Pharmacia).

Angiogenesis antibody array. Expression of angiogenesis-associated factors in the transfected cell lines was detected by the TranSignal angiogenesis antibody array (Panomics, Redwood, CA). The TranSignal angiogenesis antibody array detects 19 different angiogenesis specific cytokines. Antibodies targeting these proteins were spotted in duplicate on the array membrane. The assay was performed following the manufacturer's protocol. Four kinds of cell lysates were incubated with the antibody membranes with the same concentration of 4 mg/ml. Then biotin-labeled detection antibodies were added to the array membrane. The antibody-protein complexes on the array were visualized using streptavidin-HRP to determine which angiogenesis-related factors were activated in the sample. Chemiluminescent signal was detected using Hyperfilm ECL imaging system (Amersham Pharmacia).

Reverse transcription PCR. Semiquantitative reverse transcription PCR analysis was used to detect the mRNA expression levels of the four angiogenesis-associated factors, including hepatocyte growth factor (HGF), VEGF, placental growth factor (PIGF) and angiopoietin-1 (Ang-1). The total RNAs of two pairs of HPV16E6E7 protein positive and negative cells were extracted using Trizol solution (Promega). cDNA was synthesized with Oligo-primers with the M-MuLV reverse transcriptase (Promega). PCR reaction was carried out within a total 50 μl volume containing all the reaction components and specific primers of each gene. The expression of housekeeping gene ß-actin was performed on each sample as a control. The sequence and PCR procedure are described in Table I. The amplified products were electrophoresed in an EB stained 1% agarose gel.

Detection of HGF, VEGF, PGF and angiopoietin-1 proteins by Western blot analysis. Western blotting was performed as described previously. Equal amounts of cell extracts (100 μg) were electrophoresed. Indicated antibodies were obtained from Santa Cruz, CA, mouse monoclonal antibodies of HGF, VEGF, PIGF, and Ang-1. Immunoreaction bands were visualized by Hyperfilm ECL imaging system (Amersham Pharmacia).

Data analysis. SPSS11.5 for Windows was used for statistical analysis. The comparison of expression of HGF, VEGF, PIGF and Ang-1 in two pairs of cells was applied using Student's t-test. $P<0.05$ was considered to be statistically significant.

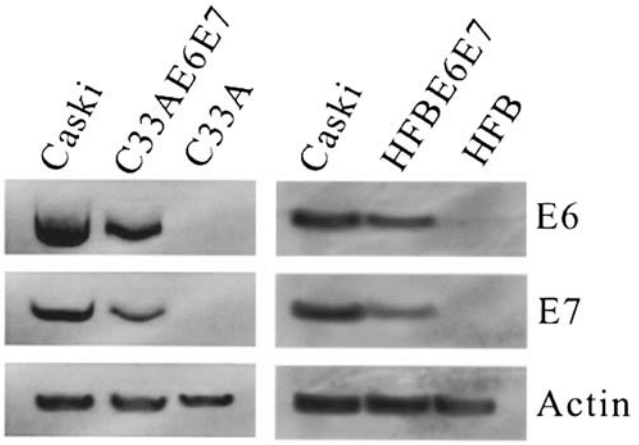

Figure 2. The expression of HPV-16 E6 and E7 proteins in C33A and HFB cells after transfection. CasKi integrates high copies of HPV-16 and express E6 and E7 proteins as a positive control. After transfection of pEGFP-HPV16E6E7, E6 and E7 proteins were expressed in C33A-E6E7 and HFB-E6E7 cells while C33A and HFB cells were HPV negative.

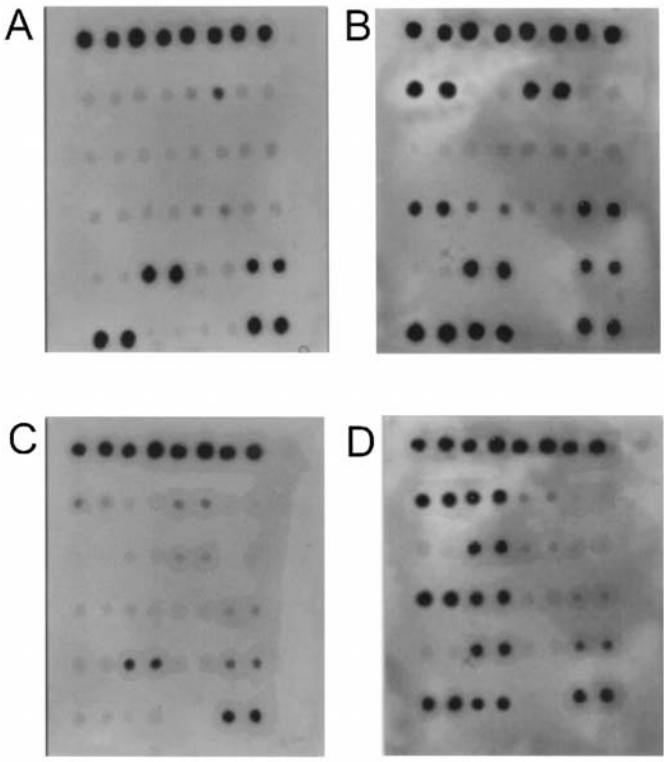

Figure 3. Expression of angiogenesis-associated factors in C33A-E6E7, C33A, HFB-E6E7 and HFB cells were detected by TranSignal angiogenesis antibody array. In the array map there were 7 angiogenesis-associated factors whose expression level was more increased (angiopoietin-1, FGFα, HGF, IL-6, IP-10, VEGF and PIGF) in C33A-E6E7 cells than in C33A cells (A and B); there were 12 factors expressed at higher level (angiopoietin-1, IL-1α, IL-1ß, IL-6, IL-8, HGF, VEGF and PIGF) in HFB-E6E7 cells than in HFB cells (C and D).

Results

Identification of primary cultured human fibroblast cells. The HFB cells were successfully obtained from anchoring human corneal tissue and verified by inverting microscope (Fig. 1A), electron microscope, and immunostaining (data not shown). The isolated fibroblasts grew and proliferated *in vitro* with typical characteristic of fibroblasts and with endocytoplasmic, ribosome, Golgy's complex and 46 chromosomes as normal diploid cells. As expression of vimentin and collagenase type I is a critical sign of fibroblasts instead of the stroma cells and lymphocytes. The deficiency of keratin distinguished the cultured HFB from the epithelial cells. The immune cytochemistry staining revealed that vimentin and collagenase type I was positively expressed while keratin was negative in the cultured fibroblasts (data not shown). Human cornea fibroblasts were separated and stably cultured *in vitro* for the following study.

Transfection of plasmid pEGFP-HPV16E6E7 into C33A and HFB cells. The construction of recombined plasmid pEGFP-HPV16E6E7 was identified by restriction endonucleases (Fig. 1B). HPV16E6E7 was successfully cloned into vector pEGFP. Then the recombined plasmid pEGFP-HPV16E6E7 was transfected into C33A and HFB cells by Lipofectamine 2000. After continuous passage in G418 containing medium, stable transfectants were selected and amplified. Green fluorescence was observed in the transfected cells under an inverted fluorescent microscopy (Fig. 1C and D).

After transfection of pEGFP-HPV16E6E7 into C33A and HFB cells, the expression levels of HPV-16 E6 and E7 proteins were detected in C33A-E6E7 and HFB-E6E7 cells by Western blotting. Our results showed that both HPV-16 E6 and E7 proteins increased greatly after transfection (Fig. 2).

Detection of angiogenesis-associated factors after transfection of HPV16E6E7. A cytokine protein expressed 3 times higher or lower in a pair of cells was considered to be of significant difference. The TranSignal angiogenesis antibody array analysis demonstrated that there were 4 cytokine proteins expressed in C33A cells including IL-8, TIMP1, VEGF and TIMP2. After transfection of HPV-16 E6 and E7, 6 other elevated cytokine proteins besides VEGF were detected. They were Ang-1, FGFα, HGF, IL-6, IP-10 and PIGF (Fig. 3A and B). In HFB cells two cytokine proteins (IL-8 and TIMP-2) were expressed before transfection of HPV-16 E6 and E7. After transfection the expression of 7 cytokine proteins increased. They were Ang-1, IL-1α, IL-1 ß, HGF, IL-6, VEGF and PIGF (Fig. 3C and D).

Thus, in the two pairs of transfected cell models, there were two common characters that could be drawn out. One is that no cytokine proteins expression decreased after transfection of HPV-16 E6 and E7 in either C33A or HFB cells. The other is that there were four common elevated cytokine proteins that could be seen in both C33A and HFB cells after transfection. They were Ang-1, HGF, VEGF and PIGF.

Increased expression of Ang-1, HGF, VEGF and PIGF in HPV16E6E7 positive cells. In order to make sure that the four common elevated cytokine proteins did increase after transfection of HPV-16 E6 and E7 in both C33A and HFB cells, we examined the mRNA and protein expression levels with traditional RT-PCR and Western blotting. As expected

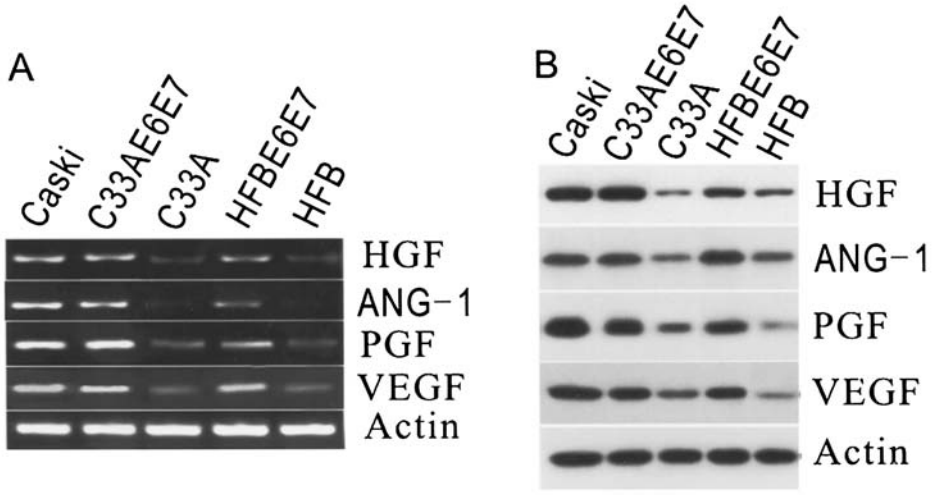

Figure 4. The expression of HGF, angiopoietin-1, PGF and VEGF mRNA and protein in C33A and HFB cells before and after transfection of HPV16E6E7. (A) RT-PCR showed higher level of HGF, angiopoietin-1, PGF and VEGF mRNA in C33A-E6E7 and HFB-E6E7 than in C33A and HFB. (B) Western blotting exhibited higher level of HGF, angiopoietin-1, PGF and VEGF protein in C33A-E6E7 and HFB-E6E7 C33A and HFB.

both the mRNA and protein levels of Ang-1, HGF, PIGF and VEGF increased in both C33A and HFB cells after transfection of HPV-16 E6 and E7 (Fig. 4A and B).

Discussion

Angiogenesis involves the activation, migration, and proliferation of endothelial cells and is regulated by several peptides and non-peptide molecules. Of these regulators, VEGF is a hypoxia-inducible angiogenic factor, and its up-regulation is thought to mediate many of the angiogenic effects of growth factors that are not direct endothelial cell mitogens. VEGF is usually expressed at high levels in most tumors including cervical cancer and has become a therapeutic target for cancer therapy (26-30). The relationship between VEGF and HPV oncoproteins has already been explained in many studies (6-10,28,29). HPV proteins contribute not only to the uncontrolled keratinocyte growth but also to the angiogenic response needed for tumor formation. HPV-16 E6 and E7 proteins in primary foreskin keratinocytes (HFKs) decreases expression of two inhibitors and increases expression of two angiogenic inducers (8). Although VEGF is crucial for blood vessel development, tumors also produce multiple non-VEGF angiogenic factors, and anti-VEGF monotherapy could potentially encounter drug resistance, suggesting that tumors could use non-VEGF angiogenic factors to grow blood vessels (31). Yet the profile of pro-angiogenic factors that contributed to angiogenesis after HPV transfection is not clear.

We transfected HPV16E6E7 into HPV negative cervical cell line C33A and HFB to investigate whether HPV-16 E6 and E7 were able to stimulate pro-angiogenic modulators. Expression of E6 and E7 protein increased after transfection of HPV-16 E6 and E7 in both cell lines. The TranSignal angiogenesis antibody array was used to screen the profile of 19 angiogenesis-associated cytokines before and after transfection. As expected a bulk of cytokines were detected to express in a higher level after transfection of HPV-16 E6 and E7 in both cell lines.

Many kinds of cytokines can stimulate or suppress the vascular formulation from different aspects. On the sheet of antibody array, C33A cells secret 4 cytokines including IL-8, VEGF, TIMP-1 and TIMP-2. After transfection of HPV-16 E6 and E7, 6 other cytokines, Ang-1, FGFα, HGF, IL-6, IP-10 and PlGF besides VEGF, were detected at higher levels. In HFB cells only 2 cytokines, IL-8 and TIMP-2, were originally expressed. After transfection of HPV-16 E6 and E7, expression of 7 other cytokines, Ang-1, IL-1α, IL-1 ß, HGF, IL-6, VEGF and PlGF besides IL-8 increased. The common cytokines increased in both cell lines were Ang-1, HGF, PIGF and VEGF after transfection of HPV-16 E6 and E7 in both cell lines. In order to verify the results of the antibody arrays, we detected the mRNA and protein level of these cytokines by traditional RT-PCR and Western blotting. Both the mRNA and protein level of the four elevated cytokines, increased in both cell lines after transfection of HPV-16 E6 and E7.

From a previous study we know that angiopoietins participate in the formation of blood vessels, which function as ligands for the endothelium-specific tyrosine kinase receptor Tie-2. Ang-1 reduces endothelial permeability of non-cerebral vessels and has a major role in vascular stabilization and maturation (32,33). PlGF is a homolog of VEGF and consists of three isoforms that arise via alternative splicing (PlGF-1, -2, and -3). This growth factor signals exclusively through VEGFR-1 (flt) and regulates cross-talk between VEGFR-2 and -1 (31-33). PlGF stimulates vessel formation and maturation *in vivo* via effects on endothelial cells, monocytes, smooth muscle, or bone marrow mobilization of circulating precursors. PlGF regulates a number of genes including Flt-1, neuropilin-2, and Egr1 and activates the serine threonine kinase Akt (34-37). In malignant tumors, HGF induces invasive, angiogenic, and metastatic responses through the c-Met/HGF receptor tyrosine kinase. In many carcinomas, HGF plays a role as a stroma-derived mediator in tumor-stroma interactions that confer invasion and metastatic potentials in cancer cells (38,39). A blockade of

HGF-Met signaling may be one strategy to inhibit tumor invasion and metastasis (40,41).

Collectively, our results demonstrate that multiple pro-angiogenic cytokines, *in vitro*, could be stimulated by HPV-16 E6 and E7 protein both in cervical cancer cell line and normal human diploid cells. Anti-angiogenesis therapy may be effective alone or in combination with biologic means aimed at E6 and E7 in the treatment of cervical cancer.

Acknowledgements

This study was supported by grants from the National Science Funds of China (No. 30500596, 30672227, 30600667, 30370657) and the '973' Program (2002CB513100). We gratefully acknowledge Dr Peter N. Jensen, Washington University, for kindly providing the cloned plasmid.

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