Human dendritic cells transfected with a human papilloma virus-18 construct display decreased mobility and upregulated cytokine production

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Received June 4, 2013; Accepted July 12, 2013

DOI: 10.3892/ijo.2013.2074

Abstract. The marked depletion of dendritic cells (DCs) in skin cancers, as well as preneoplastic and neoplastic cervical epithelium, suggests a central role for DCs in productive human papillomavirus (HPV) infection and cancer promotion. It has been suggested that HPV may facilitate tumor development by reducing DC density, contributing to a decrease in local immune surveillance. In this study, we have examined the response of human DCs transfected with a construct containing the HPV18 genome and their subsequent expression of papilloma virus proteins. Transfected cells expressed the L1 major capsid protein and upregulated E6 and E7 oncoprotein transcripts as detected by RT-PCR. Transfection of DCs also resulted in a significant increase in cytokine production. Finally, we observed that HPV18 transfection decreased the migratory activity of DCs. Our data indicate that HPV transfection of DCs leads to changes in migratory activity and cytokine production, which potentially can suppress or delay immune responses to viral antigens. Additionally, changes in cytokine production by HPV-transformed human fibroblasts and human cervical epithelial cells revealed that the migratory and antigen-presenting functions of DCs may be impaired by the suppressive effects of cytokines produced by HPV-infected epithelial and stromal cells.

Introduction

The immunological mechanisms controlling human papillomavirus (HPV) infection are poorly understood. Initial infection of basal keratinocytes does not appear to activate a local immune response and HPV-associated skin lesions can persist for months or even years in immunocompetent subjects (1). Successful viral evasion from immune controlling mechanisms is likely the result of a number of factors including the low level of viral protein expression, nuclear localization of viral proteins in the basal layer of infected epithelium and/or the absence of cytolysis or cytopathic death resulting from viral replication and assembly (2). Accordingly, there is little or no release of the proinflammatory cytokines necessary for activation and migration of antigen-presenting cells. In addition, because HPV does not undergo a systemic or viremic phase of infection, the opportunities for immune cells to capture virus and present viral antigens in the lymph nodes are limited (3).

Previous studies have described a localized immune dysfunction accompanying cervical HPV infection. This dysfunction is characterized by quantitative and qualitative alterations of $CD4^+$ T lymphocytes in the area of infection. (4,5) In addition, it has been shown that the number and distribution of Langerhans cells (LCs) are abnormal in HPV-infected preneoplastic and neoplastic cervical epithelium (6-10). LCs are included in the lineage of immature dendritic cells (DCs) (11) and are considered to be the most important professional antigen-presenting cells in the mucosal immune system (12,13).

Several paradigms have been proposed to explain LC depletion in the area of an HPV infection. For example, LC depletion might result from the creation of a locally unfavorable environment fostered by infected epithelial cells and fibroblasts. HPV-infected keratinocytes are known to express low levels of major histocompatibility complex restriction elements and do not present the necessary levels of costimulatory molecules for naive T-cell priming (14). Additionally, production of granulocyte-macrophage colony stimulating factor (GM-CSF) (15) as well as type I interferon (16,17) is significantly decreased in HPV-infected keratinocytes. Such an immunosuppressive environment might lead to a decrease in intraepithelial DC maturation and prevent migration of DCs

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Key words: papillomavirus, cytokine, dendritic cell, mobility

and macrophages to infected tissue. In addition, HPV may be capable of non-productively infecting DCs and producing a direct cytotoxic effect (7,9). Several lines of indirect evidence support this possibility. For example, DCs are located in the basal and middle third of the squamous epithelium and commonly project numerous cytoplasmic processes into more superficial cell layers (9). Therefore, the proximity of DCs to basal epithelial cells, which are considered to be the primary targets for HPV infection, could make DCs accessible for infection. Previous studies have shown that DCs express the α6 integrin receptor, which is associated with viral entry (18,19). Additionally, human monocytes and macrophages are known to be susceptible to bovine papillomavirus infection *in vitro* (20).

Spontaneous regression of flat warts is believed to be associated with activation of a cellular immune response against HPV-infected keratinocytes (21). Initiation of this response depends upon HPV antigen presentation by DCs. The contribution of HPV-infected basal keratinocytes as non-professional antigen-presenting cells is most likely insignificant in light of their low-level expression of E6 and E7 as well as their lack of the receptors necessary to mount an effective immune response (22,23). Therefore, it is possible that professional antigen presenting cells such as LCs and DCs transport HPV antigens from the skin or mucosal tissues to draining lymph nodes where the antigens are presented to specific naive T cells (11). This view is supported by studies *in vitro*, which show that DCs are capable of initiating an immune response after priming with viral proteins E6 and E7 (24). However, if this occurs, it most likely happens at late stages of infection in that seroconversion associated with high-risk forms of HPV typically takes nine months on average from the first detection of HPV DNA in a cervical biopsy (25).

Taken together, published studies indicate that DC activation is a crucial factor in anti-HPV protection. Lack of an *in vitro* system for productive infection of DCs with HPVs, prompted us to examined human DCs transfected with constructs containing the HPV genome. Our data suggest that HPV transfection of DCs leads to changes in DC migratory activity and cytokine production, which potentially may suppress or delay an immune response to viral antigens. Additionally, changes in cytokine production by HPV-transformed human foreskin fibroblasts (HFFs) and human cervical epithelial cells reveals that DC migratory and antigen-presenting functions may be impaired by the suppressive effects of cytokines produced by HPV-infected epithelial and stromal cells.

Materials and methods

Cell lines and media. The DC line RAN1 was previously generated from cells obtained from a patient with histiocytic lymphoma by using several rounds of selection and limiting dilution in order to obtain monoclonality (52). HFFs were from Cellular Engineering Technologies, Coralville, IA, USA; the HPV18-infected C4-II cell line was obtained from ATCC (ATCC® CRL1595™); and normal human cervical cells epithelial cells (NHCEC) cells were originally purchased from Bio-Whittaker (now Lonza, Inc., Allendale, NJ, USA). All cell lines, except NHCEC cells, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine and 20% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA, USA). KGM-2 medium containing epidermal growth factor, insulin, hydrocortisone and bovine pituitary extract (Lonza Inc.) was used to grow NHCEC cells.

Plasmid and transfection. The GFP Fusion TOPO cloning vector (Life Technologies, Carlsbad, CA, USA) was used to create a GFP-HPV18 plasmid containing the full length HPV18 genome. The HPV18 genome was purchased from ATCC (ATCC 45152™). Originally linearized at the unique *Eco*RI site and cloned into the pBR322 vector (53), the HPV18 genome DNA was digested with *Xmn*I and *Nhe*I, purified and ligated into the NheI-EcoRV-cut GFP Fusion TOPO cloning vector.

DCs or HFFs were seeded into 24-well plates ($2.0x10^4$ cells per well) and grown to 75-80% confluency. Transfection of cells was performed using Lipofectamine™ reagent (Life Technologies). GFP-HPV18 plasmid DNA (2 μg) and Lipofectamine reagent (10 μl) were mixed in serum-reduced medium (Opti-MEM® medium, Life Technologies) and incubated for 45 min at 21˚C. Cell monolayers, washed once with Opti-MEM medium, were overlaid with 1 ml of preformed lipid-DNA complexes. After 5 h of incubation at 37˚C in 5% CO_2, 1 ml of culture medium containing 20% FBS was added. Culture medium was refreshed 24 h after initiation of transfection. Cells were harvested at 72 h post-transfection and grown in selection medium containing G418 sulfate (Geneticin®, Life Technologies). The appropriate concentration of selection antibiotic was determined in prior experiments by titration of LD50 and varied depending on the cell line (2.5 mg/ml for DCs and 250 μg/ml for HHFs). The appearance of transfected colonies was detected by light microscopy 5-7 days after initiating selection. HHFs and DCs were transfected with GFP Fusion TOPO cloning plasmid to generate stably transfected cell lines.

RT-PCR detection of E6 and E7 transcripts. Total RNA from C4-II cells and HPV18-transfected DCs and HFFs was extracted using TRIzol® reagent (Life Technologies). DNA contamination of RNA samples was prevented by treatment with DNase (Life Technologies) according to the manufacturer's recommendation. cDNA was amplified using Super Script kit (Life Technologies) according to the manufacturer's instructions. Ten microliters of cDNA was mixed with 4 μl 10X PCR buffer, 2 μl 25 mM $MgCl_2$, 0.5 μl (100 pmol) of each primer, 32.7 μl distilled water and 0.3 μl (2.5 U) Taq DNA polymerase. The reaction mixture was then subjected to 35 cycles, each consisting of 30 sec at 94˚C, 30 sec at 50˚C and 2 min at 72˚C. The amplified products were sequenced to confirm HPV18 DNA. Primers used were: 5'-GTGAGAA ACACACCACAATACTATG-3' forward and 5'-CTCTGCGT CGTTGGAGTCGTTC-3' reverse, which amplify the E6 product of 328 bp and 5'-CGGACACACAAGGACAGGG-3' forward and 5'-GACGCAGAGAAACACAAGTATAATA-3' reverse which amplified the E7 products of 485- or 302-bp (spliced) fragments.

Immunoprecipitation and western blot analysis. Expression of HPV18 proteins was evaluated by immunoprecipitation and western blotting. HPV18-transfected and non-transfected cells

were collected after trypsinization, pelleted (1,000 rpm, 5 min) and lysed by suspension in 200 μl of PBS with 1% Triton X-100 and protease inhibitors (1:10; Complete Mini, Roche, Indianapolis, IN, USA). Cell debris was removed by centrifugation at 3,000 rpm for 5 min and supernatants were stored at -70˚C prior to use. Protein G Plus-Agarose (P-A, 100 μl; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was washed twice in PBS (pH 7.2) and incubated with monoclonal anti-L1 HPV coat protein antibody (Novocastra, Burlingame, CA, USA) or anti-E6-protein monoclonal antibody (Calbiochem, La Jolla, CA, USA) for 2 h at room temperature. Protein G Plus-Agarose-antibody complexes (P-AA) were washed twice in PBS (pH 7.2) and incubated 12 h with clarified cell supernatant at 4˚C. P-AA complexes with bound antigen were centrifuged briefly at 1,000 rpm for 5 min, washed in PBS (pH 7.2) twice and incubated with 1% SDS loading buffer for 5 min at room temperature. Agarose beads were removed by centrifugation and samples were analyzed by SDS-PAGE electrophoresis. Proteins were standardized using a modified Lowry protein assay (Pierce, Rockford, IL, USA) and was verified by Coomassie staining of the gel. Additionally, quantitative loading of proteins was determined by probing the nitrocellulose membrane with anti-actin antibodies (Santa Cruz Biotechnology). Proteins were electroblotted (15 min, 4 mA/cm^2) onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and blocked for 1 h at room temperature with 5% milk in Tris-buffered saline (TBS) and 0.5% Tween-20. After three washes, membranes were incubated with monoclonal anti-HPV L1 antibodies (1:50; Novocastra) or anti-E6-protein monoclonal antibodies (1:25; Calbiochem) for 12 h at room temperature. Antigen-antibody complexes were identified with HRP-conjugated goat anti-mouse antibodies and developed using DAB (3, 3'-diaminobenzidine) substrate (Vector Laboratories, Inc., Burlingame, CA, USA).

Cytokine ELISA. Tumor necrosis factor-α (TNF-α) interleukin-6 (IL-6), transforming growth factor-β (TGF-β) and monocyte chemotactic protein-1 (MCP-1) ELISA kits were purchased from Life Technologies. The selection of cytokines for this study was based upon published data suggesting their involvement in mechanisms controlling tumor growth or regression (14,30,36-38). Transfected and non-transfected cells (10^6 cells/well) were placed in 12-well plates and grown to confluency and activated with lipopolysaccharide (1 μg/ml; LPS; Sigma Chemical Co., St. Louis, MO, USA). Forty-eight hours after addition of LPS, growth medium was collected and stored at -80˚C until analyzed.

Southern blot analysis. An HPV18 ^{32}P-end-labeled DNA probe was generated using a DNA labeling kit (Ready-To-Go, GE Healthcare Life Sciences Inc., Piscataway, NJ, USA), 50 μCi [α^{32}P]-dCTP and ~50 ng of HPV18 plasmid DNA. After a 15-min incubation at 37˚C, MicroSpin G-50 columns (GE Healthcare Life Sciences Inc.) were used to purify the labeled DNA. A DNA isolation kit (Puregene® DNA, Qiagen, Valencia, CA, USA) was used for extraction of DNA from HPV-transfected cell lines. Approximately 10 μg of DNA was digested with *Eco*R1 restriction enzyme (New England Biolab; Beverly, MA, USA) and resolved on a 1.0% agarose gel. Denatured DNA (0.25 N HCl for 10 min) was transferred by capillary to nylon membranes (ZetaProbe Nylon, Bio-Rad). The membranes were incubated 14 h at 55˚C with labeled HPV18 DNA (5 ng) in 10 ml of hybridization buffer [1.5X SSPE, 7% SDS, 10% (wt/vol) polyethylene glycol 4000]. The blots were washed twice for 15 min each at room temperature with 2X SSC-1% SDS and then twice with 0.1X SSC-1% SDS for 25 min each at 55˚C. The blots were then exposed to X-ray film for 15 h at -80˚C.

Northern blot analysis. In order to recover total intracellular RNA, cells were washed with PBS (pH 7.2) and lysed with TRIzol reagent according to the manufacturer's instructions. RNA (20-50 μg) was denatured for 15 min at 70˚C in RNA buffer [50% formamide, 5% formaldehyde, 1X morpholinepropanesulfonic acid (MOPS)], separated on a 3% formaldehyde-agarose gel and transferred to a nylon membrane (ZetaProbe Nylon). Membranes were hybridized with ^{32}P-labeled plasmid for 14 h at 60˚C and washed as described above. A ^{32}P-labeled 18S ribosomal RNA probe (Ambion, Austin, TX, USA) was used to standardize loading.

Colony formation assay. HPV18 transfected and non-transfected cells (10^6/well in 12-well plates) were placed in methylcellulose medium (3 ml; Life Technologies) containing 20% FBS (Atlanta Biologicals) and L-glutamine (Life Technologies). After 14 days of culture, colonies were counted under light microscopy.

Chemotaxis assay. The *in vitro* migration of DCs was assayed in 24-well transwell cell culture chambers (54). DCs were washed and suspended in DMEM, supplemented with 20% FBS. Filter inserts (8 μm) were placed into the cell culture plate wells to create upper and lower compartments. DCs (5x10^4) were applied into the upper chamber, while the lower chamber was filled with 200 μl of medium (DMEM + 20% FBS) containing 50 μg of GM-CSF. After 4 h of incubation (37˚C, 5% CO_2), cells in the upper side of the filter were removed and filters were scraped to remove residual cells. Filters were fixed (3:1 methanol/acetone mixture, 5 min) and cellular nuclei were stained with hematoxylin. Five random microscopic fields were counted per filter to evaluate the number of migrated cells.

Statistical analysis. Statistical significance was assessed by the Student's paired t-test and considered significant at $P<0.05$.

Results

Characterization of HPV18-transfected DCs. Because of difficulties in propagating papillomaviruses and generating infectious virus *in vitro*, we used DNA transfection to deliver viral DNA into DCs. One week after transfection, colonies of DCs were isolated and subcultured for an additional 2-3 passages. A stably transfected DC line, which persistently maintained the HPV18 genome, was utilized for experiments reported herein.

It has been reported that HPV DNA regularly becomes integrated into host cell DNA (1). Therefore, it was of interest to determine if our HPV18 DNA construct is maintained as a plasmid or integrated into the genome. Total DNA from trans-

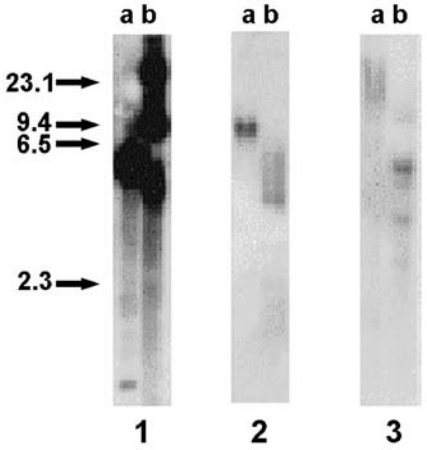

Figure 1. Southern blot analysis of transfected cell cultures demonstrating integration of HPV18 genomes. Total genomic DNA (10 μg) was not digested (column a) or digested with *Eco*R1 restriction enzyme (column b) linearizing the 8.0 kb HPV18 genome. Lane 1, total DNA from HPV18 transfected DC; lane 2, total DNA from HPV18 transfected HFF; lane 3, total DNA from HPV18 transformed cervical keratinocytes.

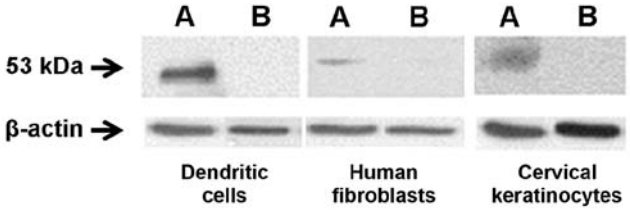

Figure 2. Western blot analysis of L1 protein expression in HPV transfected and non-transfected cells. (A) Transfected cells. (B) Non-transfected control.

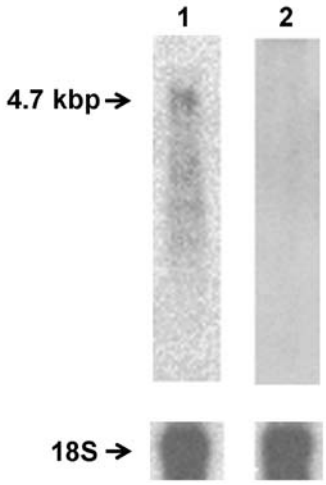

Figure 3. Northern blot analysis of L1 transcripts in HPV18 transfected DCs. Membranes were hybridized with ^{32}P-labeled HPV18 plasmid from HPV transfected DC (lane 1) and non-transfected DCs (lane 2). A ^{32}P-labeled 18S ribosomal RNA probe was used to standardize loading.

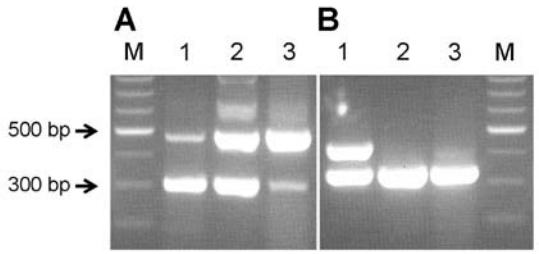

Figure 4. Detection of HPV18 E6/7 transcripts in HPV18 transformed cells. Total RNA from HPV18-transformed C4-II cells and HPV18-transfected DCs and HFFs was collected and used to detect E6 and E7 transcript by RT-PCR. E6 transcript amplification (A). E7 transcript amplification. (B) Lane 1, RNA from HPV18 transfected DC. Lane 2, RNA from HPV18 transfected HF cells. Lane 3, RNA from C4-II cells. Lane M, marker.

fected DCs was isolated as described in Materials and methods. Southern blot analysis revealed integration of HPV18 DNA into the DC genome; no extra-chromosomal plasmid could be detected in DNA samples isolated from transfected cells following cleavage with *Eco*RI (Fig. 1, lane 1). Transfection did not induce differentiation of DCs to a more mature or macrophage-like phenotype or change their growth pattern (data not shown).

L1 is the major HPV capsid protein and is highly conserved among all papillomaviruses. Since expression of papillomavirus L1 protein is important for development of an anti-HPV immune response (26-29), we sought to establish if HPV18-transfected cells express this protein. Immunoprecipitation and western blot analysis established that L1 protein was present only in extracts of transfected cells (Fig. 2). To confirm these data, we further analyzed extracts for the presence of L1 mRNA. Total RNA from transfected and non-transfected cells was collected and subjected to northern blot analysis. Consistent with our western blot results, HPV L1 specific transcripts were detected only in HPV18 transfected DCs, whereas no HPV RNA was found in non-transfected DC control (Fig. 3).

It is well documented that HPV18 E6 and E7 proteins are important in cell transformation; therefore, transcription of E6 and E7 genes was analyzed by RT-PCR. Amplicons consistent with the expected RT-PCR product of the E6 and E7 gene (~302 and 485 bp for E6 and ~328 bp for E7) were detected in transfected DC cells (Fig. 4A, lane 1 and B, lane 1). Sequencing of the excised bands confirmed these results.

In view of the possibility that expression of viral transcripts or proteins could influence immune functions, IL-6, MCP-1, TGF-β and TNF-α expression was analyzed in transfected and non-transfected DCs. Selection of cytokines was based upon previous reports on their importance in HPV-associated cervical neoplasia (30,31) or their *in vitro* expression following DC exposure to HPV proteins (24,32). To determine the effect of HPV18 transfection on cytokine production, transfected DCs, non-transfected DCs and DCs transfected with empty vector were cultured to confluency. As a positive control for cytokine production, LPS (1 μg/ml) was added to the medium of both transfected and non-transfected cell lines. Transfection with the empty vector did not influence cytokine production (data not shown). IL-6, TGF-β and TNF-α production was significantly increased in supernatants of HPV18-transfected DCs compared to non-transfected controls (Fig. 5A-C). LPS stimulation resulted in upregulation of IL-6 and TNF-α by both non-transfected and transfected cells; however, the increases in IL-6 and TNF-α production following LPS

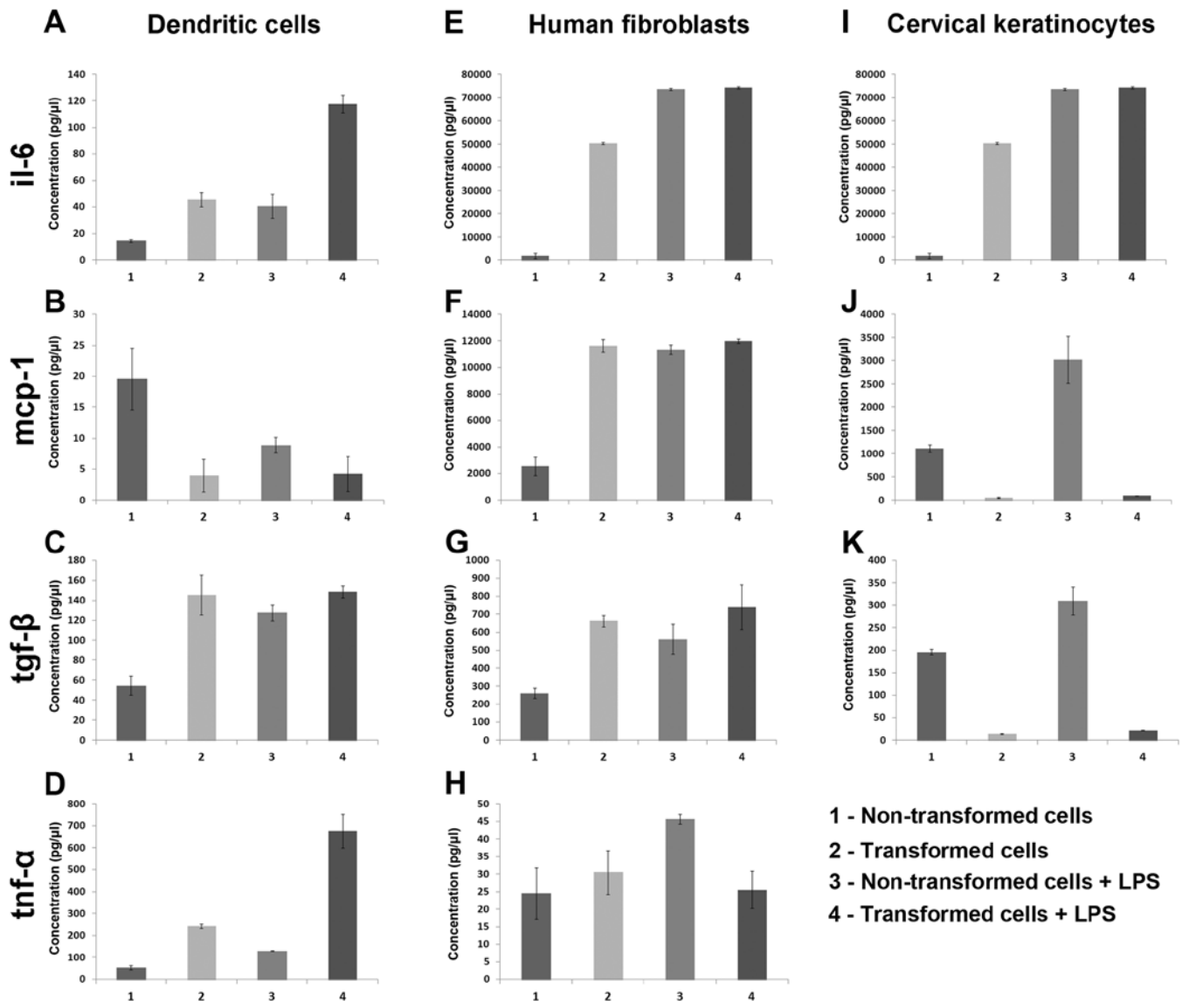

Figure 5. Analysis of cytokine production by HPV18 transfected and non-transfected cells. Transfected and non-transfected cells were placed in 12-well plates, grown to confluency and activated as indicated by the addition of lipopolysaccharide. Growth medium was collected and used to determine cytokine/chemokine concentration by ELISA.

stimulation were greater in HPV-transfected cells than in non-transfected controls. LPS treatment of non-transfected DCs increased TGF-β production, as did transfection with the HPV construct. However, LPS stimulation of HPV18 transfected DCs did not cause further upregulation of TGF-β compared to non-transfected cells. The concentration of MCP-1 was significantly decreased in supernatants of HPV-transfected DCs versus non-transfected cells (Fig. 5B).

Migration of DCs from the site of infection to the regional lymph nodes is essential for induction of an effective immune response; therefore, changes in the migratory function due to HPV infection, may potentially impact the outcome of an anti-HPV immune response. To determine if HPV18 transfection might influence the migration of DCs, we conducted migration assays using a Boyden microchemotaxis chamber. Chemotaxis was significantly enhanced by addition of GM-CSF to the medium (Fig. 6). Spontaneous motility of DCs transfected with HPV18 was significantly higher than that of non-transfected cells. However, the chemotactic response of transfected DCs was significantly lower than that of non-transfected cells, although it remained higher than the response to non-conditioned medium. The presence of the empty vector control did not affect DC migration (data not shown).

Characterization of human foreskin fibroblasts (HFF) transfected with HPV18 DNA and HPV18-infected human cervical epithelial cells (C4-II). HPV18 infects basal epithelial cells of the cervix, therefore, cytokines produced by cervical epithelial cells and fibroblasts may influence the function of local DCs. For this reason, we sought to determine if HPV18 transformation promoted cytokine production in human fibroblasts and cervical epithelial cells. The HPV18-infected human cervical epithelial cell line (C4-II) has been shown to maintain an expression profile that accurately represents cervical cancer when compared to normal cervix biopsies (33). Therefore, this cell line was used to evaluate the effects of virus transforma-

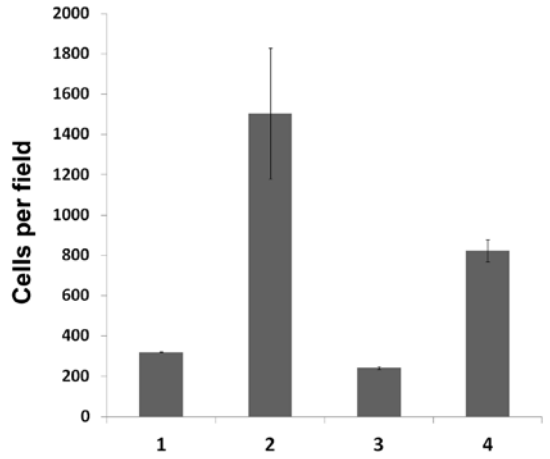

Figure 6. Analysis of migratory activity of HPV18 transfected DCs. DCs ($5x10^4$) were placed into the upper compartment of the Boyden chamber, while the lower chamber was filled with culture medium containing GM-CSF. After 4 h of incubation, cells in the upper side of the filter were removed and filters were scraped to remove residual cells. Filters were fixed and cellular nuclei were stained with hematoxylin. Five random microscopic fields were counted per filter to evaluate the number of migrated cells. Bar 1, spontaneous migration of non-transfected DCs; bar 2, migration of non-transfected DC in the presence of GM-CSF (50 ng/μl); bar 3, spontaneous migration of HPV18 transfected DCs; bar 4, migration of HPV18 transfected cells in the presence of GM-CSF (50 ng/μl).

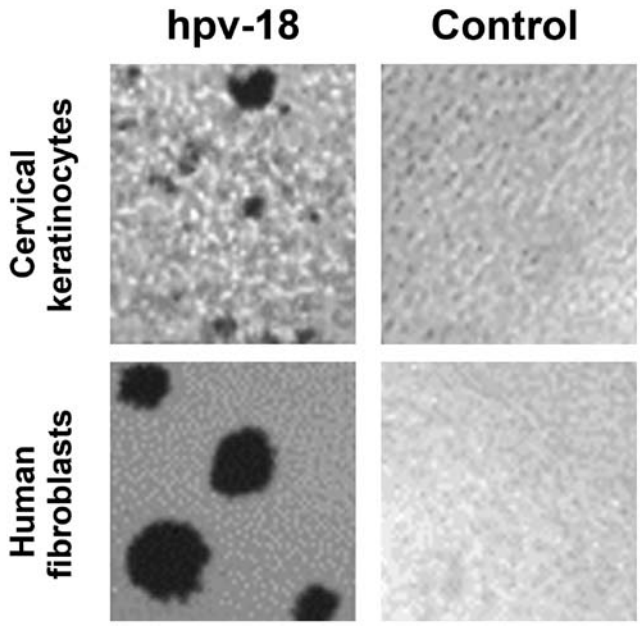

Figure 7. Analysis of anchorage independent growth of HPV18 transformed HF and cervical keratinocytes. HPV18 transfected and non-transfected were placed in methylcellulose medium containing 20% FBS and L-glutamine. After 14 days of culture, colonies were counted under light microscopy.

tion on cytokine expression and growth patterns of cervical epithelial cells. Normal human cervical epithelial cells (NHCEC) were used as a control.

Human fibroblasts were transfected with HPV-GFP plasmid and cultured for selection of stably transfected cell colonies. A transformed cell line (HPV18-HFF) that persistently maintained the HPV18 genome was isolated and used in all further experiments. Southern blot analysis revealed genomic integration of viral DNA in transfected HFFs that was consistent with that observed in HPV18-infected C4-II cells (Fig. 1, lane 2). Additionally, transfected HFFs and HPV18-infected C4-II cells both expressed HPV18 L1 protein (Fig. 2), as detected by immunoprecipitation and western blotting.

In order to determine if transcriptional activation of the E6 and E7 genes could be detected in transfected HFFs and transformed C4-II cells, we again performed RT-PCR as described above. Consistent with our observations for DCs, transcriptional activation of the E6 and E7 genes was identified in HFFs and C4-II cells (Fig. 4). Significantly, HPV18 transfection resulted in transformation of HFFs as suggested by the colony formation assay (Fig. 7). Transfected HFFs were able to support anchorage-independent growth in soft agarose medium. In contrast, non-transfected HFFs failed to produce colonies in soft agarose. It has been reported that expression of E6 and E7 genes affect cell growth properties such as saturation density and colony formation in soft agarose (34,35), consistent with our observations.

In view of our observation that HFFs and cervical epithelial cells were able to support the expression of some HPV18 proteins, we sought to determine if transfection with the HPV18 construct would influence cytokine production. Culture media from HPV18-transformed (HPV18-HFFs and C4-II) and non-transformed cells (HFFs and NHCEC) was analyzed for cytokine production by ELISA. HFFs transfected with empty vector were used as a negative control in all experiments and LPS treatment was used as a positive control. When compared to non-transfected cells, those transfected with empty vector consistently showed no difference in cytokine production (data not shown).

When compared to non-transformed cells, all transformed cells showed statistically significant differences in cytokine production (Fig. 5), with the greatest difference being a 60-fold increase in IL-6 production (Fig. 5E and I). Interestingly, transformed keratinocytes were refractory in MCP-1 and TGF-β production, even upon LPS stimulation, when compared to their non-transformed counterparts (Fig. 5J and K). Additionally, upon LPS stimulation, transformed fibroblasts displayed decreased TNF-α production when compared to non-transformed cells (Fig. 5H).

Discussion

In order to investigate the possible consequence of HPV infection on DC functions we used transfection as a form of viral genome delivery. Transfection of DCs and expression of viral proteins did not result in cell death; conversely, we were able to develop several lines of DCs containing the complete HPV18 genome. Expression of L1 major capsid protein was revealed in western blot analyses, as were L1 transcripts by Southern blotting. Although evidence of E6 or E7 protein production was lacking in transfected DCs, all transfected cell lines expressed HPV18 E6 and E7 transcripts, suggesting possible low-level protein expression below the sensitivity of our detection method. Expression of the L1 protein by DCs may be important for stimulation of an antibody response

against HPV. Indeed, Steele and Gallimore (28) observed the production of specific antibodies during the course of HPV infection, the majority of which were antiviral capsid proteins. They further reported that as papillomas progressed to carcinomas, there was a marked increase in the response to L1 and L2 suggesting a change in interaction between virus-infected host cells and the host's immune system (26,27). However, it is believed that antibodies to capsid proteins are not sufficient to totally suppress infection (30) because of the limitation of HPV capsid protein expression to superficial epithelial cells, which do not represent the site of virus replication (3).

In our experiments, HPV18 transfection of DCs resulted in increased production of cytokines involved in DC migration. This phenomenon was accompanied by increased spontaneous migration of DCs after HPV18 transfection compared to non-transfected cells, suggesting that HPV18 transfection could activate cellular mechanisms controlling DC migration. It has been shown that TNF-α and IL-6 contribute to the migration of DCs through the dermis *in vivo* and *in vitro* (36-38). Constitutive and inducible expression of IL-6 by DCs could facilitate the induction of cutaneous immune responses (36). Importantly, DCs are reported to be a significant source of IL-6 in lymph nodes during the primary immune responses to cutaneous antigens (39). Our data show that HPV18 transfection increases production of TNF-α and IL-6 by DCs. This result suggests that HPV18-transfected DCs are potentially capable of inducing an immune response to viral proteins as a result of increased migratory activity and upregulation of cytokines involved in antigen priming of T cells in lymph nodes. Additionally, HPV-transfected DCs released significantly more inflammatory cytokines (TNF-α and IL-6) after LPS stimulation than non-transfected cells. These data suggest that papillomavirus may act as a cofactor for induction of proinflammatory cytokines in DCs when they are exposed to secondary inflammatory stimuli.

Keratinocytes are the primary target for papillomavirus infection (23). Previous studies have shown that keratinocytes secrete a variety of lymphokines that are critical components of the host response to infection or injury (40,41). HPV infection may result in changes in cytokine production creating a favorable environment for viruses to evade clearance by immune cells (3,14). In this study, we found that cervical epithelial keratinocytes and HFFs support expression of viral proteins. Although, HPV18 transfection altered cytokine production in keratinocytes and HFFs, these changes were cell-type specific.

Since several reports proposed MCP-1 and TGF-β involvement in the pathogenesis of HPV-associated cervical neoplasms (30,31), we studied production of these cytokines by HPV transfected cells. HPV18 infection of cervical keratinocytes significantly decreased production of MCP-1. Previous studies showed that MCP-1 plays an important role in recruitment of monocytes to areas of virus entry and activates these cells to secrete a variety of cytokines and chemokines (42,43). It is believed that monocytes attracted into infected tissue can differentiate into a variety of cells, including DCs (44). Thus, a local decrease in MCP-1 might alter the mechanism of DC recruitment into tumor tissue leading to a decreased number of local DCs. Kleine and coworkers (45) reported rapid growth of tumors in nude mice inoculated with MCP-1-negative HeLa cells and suggested that this effect was a result of a decrease in macrophages migrating into the tumor tissue due to low levels of MCP-1 produced by the tumor cells.

Decreases in TGF-β production by transformed keratinocytes may also contribute to long-term maintenance of papillomavirus infection. TGF-β is an important factor in differentiating HPV-infected keratinocytes. For example, TGF-β treatment of cultured monolayers is accompanied by downregulation of HPV E6 and E7 transcription (46). Treatment of organotypic cultures with TGF-β results in renewed ability of keratinocytes to express morphological and biochemical markers, characteristic of a more normal and differentiated phenotype (47). Previous studies established that TGF-β stimulation is requisite for the development of DCs *in vitro* and *in vivo* (48,49) and is involved in the reciprocal control of DC chemotaxis (50). In addition, TGF-β inhibits upregulation of critical T-cell costimulatory molecules on the surface of DCs. Furthermore, TGF-β appears to enhance antigen processing and costimulatory functions of epithelial LCs (reviewed by Strobl and Knapp) (51). Thus, a decrease in local TGF-β as a result of HPV infection may abrogate mechanisms of immune recognition and/or stimulation and suppress DC migratory ability.

The reaction of human HFFs to HPV18 transformation differed markedly from that of cervical keratinocytes. For example, HPV18-HFFs produced significantly more MCP-1 and TGF-β than non-transformed cells, whereas cervical keratinocytes responded in the opposite manner. The increases in cytokine production by HPV18-HFFs suggest that HPV transformation may lead to the development of a localized tissue environment supportive for increased DC numbers and differentiation. HFFs are a main component of the subepithelial stroma. Previous studies have shown that the immunological reactions of tumor tissue and peritumoral stroma may be different. For example, Beutner and Tyring (22) found that, if immature DCs are predominantly localized in breast carcinoma tissue, mature DCs adhere selectively to peritumoral areas. Mota and coworkers (14) discovered that $CD1^+$ DCs were occasionally detected in cervical intraepithelial neoplastic lesions; however, leucocytes with an activated phenotype accumulated immediately below the epithelial lesions in subepithelial stroma (24). These observations and data presented here, are consistent with the proposal that HFFs expressing papillomavirus proteins develop an immunoactivating cytokine profile; nevertheless, additional investigations are necessary to delineate the role of stromal HFFs in pathogenesis of papillomavirus infection.

Acknowledgements

Work conducted in the laboratory of V.C.L. was supported, in part, by an award from the U.S. Department of Health and Human Services, HERSA award C76HF19321; work conducted in the laboratory of A.A.R. by FCP No. 14.A18.21.1930, Russian Federation President Grant for young scientists (Dr.Sci.) МД-433.2013.4, as well as the Federal Center of Collective Use and Pharmaceutical Research and Education Center, Kazan (Volga Region) Federal University, Kazan, Russia.

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