A human vascular endothelial cell model to study angiogenesis and tumorigenesis

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Endothelial cell biology has recently been the subject of considerable interest in thrombosis and cancer research. However, the successful establishment of immortalized human endothelial cells which retain differentiated cell characteristics has been rare. We have successfully established immortalized human umbilical vein endothelial cells (HUVECs) by human papilloma virus (HPV)-16 E6-E7. HPV-16E6, E7 and E6-E7 were successfully introduced into HUVEC cells. Both E6 and E7 cultures had an extended lifespan but eventually underwent senescence. E6-E7 cultures 4–5–2G, however, acquired an indefinite lifespan in culture but did not undergo malignant conversion. Telomerase activity was not detected in either E6 or E7 cultures; however, telomerase was detected in E6-E7 4–5–2G cells. The cells exhibited a ‘cobbledstone’ morphology and developed a capillary-like tube structure upon reaching confluence. The 4–5–2G line expressed Factor VIII related antigen and took up DiI-Ac-LDL as markers of endothelial origin. The line expressed integrin subunits (αvβ3, αvβ5,β1, α2, α3, β4 and α6) consistent with an endothelial origin. The higher passage of 4–5–2G line showed a similar intensity of integrin immunostaining to that of primary HUVECs. Subsequent infection of these immortal cells with the Kirsten murine sarcoma virus which contains an activated K-ras oncogene induced morphological transformation that led to the acquisition of invasion capability and neoplastic properties. Telomerase was also detected in the tumorigenic v-Ki-ras transformed cell line. These cell lines should be useful for studies of the molecular mechanisms underlying normal and neoplastic endothelial cell proliferation and migration, and might also provide an in vitro model for development of pharmacologic and gene therapy for cardiovascular thrombosis and cancer.

Introduction

The proliferation and function of endothelial cells is important in such diverse processes as development, wound healing, cardiovascular thrombosis and neoplasia (1–6). Endothelial cell proliferation also occurs with neoplastic transformation in Kaposi’s Sarcoma, which is a prevalent neoplasm affecting patients with the human immunodeficiency virus (7–11). Thus, endothelial cells have recently received considerable attention as a potential target for pharmacologic and gene therapy for cardiovascular disease and cancer (12–24).

The repertoire of integrin cell adhesion molecules expressed by endothelial cells have been recognized as an important molecular target for the development of novel biologic therapy, since certain integrins have been implicated in the control of endothelial cell proliferation, apoptosis, and migration (18–24). The integrins are heterodimers comprised of different α and β subunits that form a superfamilly of cell surface receptors involved in cell–cell and cell–extracellular matrix adhesion and recognition. Endothelial cells have been shown to selectively express integrin heterodimers such as αvβ3, α2β1 and α6β4 during the formation of neovascularisation in human neonatal tissues (19). Endothelial cell proliferation induced by various angiogenesis factors and integrin ligands is associated with increased expression of these integrins and migration (20–23). Antagonists of the αvβ3 integrin receptor have been shown to specifically inhibit neoangiogenesis induced by angiogenesis factors and neoplasms, and can inhibit tumor growth by induction of apoptosis of endothelial cells (21). Integrin αvβ3 antagonists have also been shown to effectively inhibit thrombosis in experimental models of cardiovascular disease (24).

Continuous cell lines derived from endothelium would be useful not only in the mechanical analysis of endothelial cell growth but also in the development of new antiangiogenesis or antimetastasis therapy. The generation of human endothelial cell lines either by spontaneous transformation (25) or by the introduction of oncogenic viral DNA (mostly SV40 except one) has been reported (26–30). In most cases, the endothelial specific production of Factor VIII related antigen is lost, whereas other endothelial cell markers are retained. The expression of integrins by these immortalized endothelial cells has not been reported. Thus, the successful establishment of immortalized human endothelial cells which fully retain differentiated cell characteristics has been rare.

Since human papilloma virus (HPV-16*) E6 or E7 alone has been known to be capable of immortalizing certain human cells (31–35), we have attempted to immortalize primary human umbilical vein endothelial cells (HUVECs) with E6 and E7 as well as E6-E7 genes. In doing so, we have successfully established immortalized but nontumorigenic HUVECs by HPV-16 E6-E7. E6 or E7 alone extended the life span but eventually underwent senescence. In order to understand the mechanism of senescence, we have examined their telomerase activities. Telomerase activity was not detected in either E6 or E7 cultures; however, telomerase was detected in E6-E7 immortalized line (4–5–2G). We describe the generation and characterization of immortalized HUVECs by HPV-
16 E6-E7 genes, and show that they retain important phenotypic markers of primary HUVEC, namely Factor VIII related antigen and integrin cell adhesion molecule subunit expression. In addition, we demonstrate the subsequent conversion of such non-tumorigenic but immortalized HUVEC cells into tumorigenic cells by the introduction of an activated Ki-ras oncogene.

Materials and methods

Cell culture and media

Proliferating secondary normal HUVECs were obtained from Clonetics, San Diego, CA. Growth and maintenance medium for these cells consisted of the endothelial cell growth medium (EGM) (Clonetics, San Diego, CA) with 5% fetal bovine serum (FBS). EGM is based on the MCDB 131 formulation supplemented with the following: 10 mg/ml human recombinant epidermal growth factor, 10 µl/ml hydrocortisone, 50 µg/ml gentamycin, 50 ng/ml Amphotericin B and bovine brain extract, −12 µg/ml final protein concentration and also contained 10 µg/ml heparin.

Retroviral infection of HUVECs

PA317 amphotropic packaging cell lines stably transfected with pLXSN 16 E6 (HPV-16 E6), pLXSN 16 E7 (HPV-16 E7) or pLXSN 16 E6-E7 (HPV-16 E6E7) (provided kindly by D.Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA) were grown to 70–80% confluence, and supernatants were collected for 24 h and stored in aliquots at −80°C. One-day-old cultures of secondary HUVECs (plated at 105 cells per dish) were infected with 200 µl of virus stock in 2 ml of medium containing Polybrene (Sigma, St Louis, MO) at 4°C for 1 h. The virus was then removed and the medium replaced with EGM + 5% FBS. Selection medium, EGM with 200 µg/ml G418 (Gibco, Gaithersburg, MD) was added after 72 h. Cultures were maintained in selective media for 7 days. The G418-selected transformed cells were then grown in EGM + 5% FBS and further expanded. At this stage, part of the culture was stored by cryopreservation and another part was used for long-term propagation.

Telomerase assay

Cell extracts were prepared and assayed for telomerase activity by the telomeric repeat amplification protocol (TRAP assay) as described (37). After electrophoresis in 10% polyacrylamide gels in 0.5 X TBE buffer (38), the amplified hexanucleotide repeat pattern characteristic of telomerase activity was visualized by autoradiography. An average exposure time of 24 h at −80°C was used. Authenticity of the telomerase ladder signal was confirmed by assaying in parallel DNAaase treated cell lysates (telomerase extracts were mixed 1:1 with DNAaase free RNAaase 30 min prior to addition of cell extract to the reaction mix). The protein content in cell extracts was determined by the Bradford method (39) using reagents from Bio-Rad Laboratories (Munich, FRG). Optimal protein concentrations for cell lines, which just saturate the PCR reaction, but do not inhibit the Taq-DNA polymerase (40) were found to be 0.1–1.0 µg/µl TRAP reaction. To allow comparison of telomerase activity among cell lines, identical amounts of total cellular protein were assayed under the same conditions. 0.1 µg total cellular protein of the LNCaP prostate cancer cell line, which gives a very prominent and extensive ladder signal, was used as a positive control for each set of probes examined. This cell line has been previously reported to express telomerase (41). A lysis buffer blank and RNAaase pretreated samples were used as negative controls. Three independent experiments were performed for each cell line and cells found negative were then mixed with the high telomerase expressing LNCaP prostate cancer cell line extract to eliminate the possibility of the presence of inhibitors of Taq-DNA polymerase in the negative cell extracts.

Telomere restriction fragment (TRF) length analysis

Genomic DNA was isolated and made free of RNA by the method of Silhavy et al. (42). The DNA was digested by Hinfl according to the manufacturer’s procedure, run on agarose gels, and blotted onto NYTRAN membranes as described (38). A probe for telomeric repeat sequences was prepared by radiolabelling synthetic oligonucleotides containing N(TTAGGG) repeats of the sequence using T4 polynucleotide kinase. The filter was hybridized to the probe and washed as described (38) and exposed to Kodak Biomax Film. The autoradiogram was scanned with a laser densitometer (Molecular Dynamics, Inc., Sunny Valley, CA). The digitized image was used to calculate mean TRF length using the formula L = 2OD2/(OD1+L1) where OD2 is the integrated signal at position 1 and L1 is the length of DNA fragments at position 1. The range of sizes from 2–20 kb was used in the calculations.

Demonstration of Factor VIII-related antigen

The staining for Factor VIII-related antigen was carried out using rabbit anti-human Factor VII-related antigen and an immunocytochemistry kit, Peroxidase (DAKO, Carpinteria, CA).

Uptake of acetylated low density lipoprotein (Di-Ac-LDL)

HUVECs grown on glass cover slips were incubated for 4 h at 37°C in medium containing 10 µl/ml AC-LDL labeled with 1,1-dioctadecyl-3,3,3'-tetramethylrhodo-carboxyanine prechorolite (Di-Ac-LDL, Biomedical Technologies, Inc. Stoughton, MA). To visualize the Di-Ac-LDL uptake, the cells were washed three times with PBS, sealed under coverslip in 90% glycerol and visualized using standard rhodamine excitation.

Chromosome analysis

Karyological analyses were carried out by Drs J.Kaplan and B.Hukkan, Children’s Hospital of Michigan, Detroit, MI.

Tumorigenicity in nude mice

Cells (1×107) in 0.2 ml of phosphate-buffered saline (PBS) were injected s.c. into the mid-dorsal intrascapular region of adult 129J nude mice to determine tumorigenicity.

Antibodies

The derivation and specificity of the murine monoclonal antibody UM-A9 for the β1 subunit of the α6β4 integrin has been previously described (43–45). Murine monoclonal anti-integrin subunit specific antibodies for β1 (PAC10), α2 (P1E6), and αvβ5 (P1F6) were obtained from GibCO, BRL, (Grand Island, NY); for α3 (P1BS) (46) was obtained from Telios Inc. (San Diego, CA); and for αvβ3 (LM609) was a gift from Dr Shaker Mousa (DuPont/Merck, Wilmington, DE). Anti-α6 rat monoclonal antibody GoH3 (47) was obtained from Biosource International (Camarillo, CA). Appropriate species and sub-class antibodies were used as controls in all experiments.

Immunofluorescent staining and flow cytometry

The relative level of surface integrin expression of the HUVEC lines was compared by immunofluorescence staining and flow cytometry. Briefly, 106 cells were aliquoted in each tube and incubated with normal goat serum (10%) final concentration) at 4°C for 10 min for blocking of nonspecific binding sites. The quantity of antibody needed to saturate available sample sites and provide maximal staining was determined in preliminary experiments. 1 µg purified antibody or 2 µl mouse ascites containing mouse or rat anti-human integrin monoclonal antibody was added to each tube and incubated at 4°C for 30 min. The same amount of mouse or rat Ig subclass-matched monoclonal antibody was used as a control (mouse IgG, Sigma, St Louis, MO; and rat IgG, Dako Corp., Carpinteria, CA). After incubation, cells were washed, resuspended in 100 µl of PBS with 2 µl of R-phycocerythrin (PE) conjugated goat anti-mouse or fluorescein isothiocyanate (FITC) conjugated goat anti-rat IgG heavy and light chain antibody (Molecular Probe, Inc, Eugene, OR), and incubated at 4°C for 30 min. Then cells were washed, and analyzed by a flow cytometer (FCAscan, Becton Dickinson, San Jose, CA) calibrated with standard beads at an excitation wavelength of 488 nm. The log fluorescence intensity of 105 cells was collected and plotted.

Southern blot analysis

DNA blot analysis was performed as described (48). High molecular mass DNA was digested with EcoRI and applied to a 1.0% agarose gel. After electrophoresis, the DNA fragments were transferred from the gel to a nitrocellulose membrane and hybridized to a random-primed 32P-labeled DNA probe, HPV 16 E6-E7 gene (56–879 nucleotides) (36) was used as probe.

Immunoprecipitation of HPV-16 E6, E7 and p53 ras proteins

Exponentially growing cells were labeled using the NEN 35S met cys express kit for 4 h at 0.15 µCi/ml. The cell lysates were immunoprecipitated with anti E6 (provided by V.Band), anti-E-7 (Triton) and anti-ras p21 (Y13-259, Oncogene Science) and electrophoresed on a 15% polyacrylamide gel, as described (49).

Invasion assay

Forty thousand cells were evenly loaded onto Becton Dickinson Invasion Chambers (Becton Dickinson) containing 0.5 ml EGM/0.1% BSA in the upper chamber and 0.75 ml EGM/0.1% BSA in the lower chamber respectively. After incubation for 18 h in a 5% CO2 incubator, cells were removed from the upper surface of the membrane with a cotton swab. Cells on the lower surface were fixed in methanol, stained with Giemsa, and counted under a microscope.

Results

Extended life span of HUVECs by HPV-16 E6 or E7

Since HPV-16 E6 or E7 alone efficiently immortalized certain human cells (31–35) it was of interest to determine whether
HUVECs showed a similar or distinct requirement for immortalization. We introduced HPV-16 E6, E7 or both E6-E7 into secondary HUVEC by retroviral infection. After 48 h cultures were transfected to EGM + 5% FBS and were grown for 10 days in a selective medium, containing G418, allowing the formation of neomycin-resistant clones and selected for outgrowth of immortal cells. A number of clones were isolated and stored frozen in liquid nitrogen. Seven of each group were further characterized. Normal HUVEC senesced after a limited lifespan in vitro within 10 passages. In contrast, HPV-16 E6 and E7 transformed HUVEC showed a lifespan extension of 9–10 passages which has been referred to as the M1 stage of immortalization (35). This lifespan extension was longer for E6-transferred HUVEC than E7 HUVEC. Both E6 and E7 HUVEC cultures eventually underwent senescence at 18–20 passages and 4–5 month following infection. However, one clone, designated E6-E7 HUVEC 4–5–2G, has an apparently unlimited lifespan and has been successfully subcultured for >30 passages over the course of 1 year with no evidence of decreased proliferative capacity. These lines were further characterized.

Telomerase activity in HPV-16 E6-E7 immortalized cell line
Immortalization of human cells has often been associated with telomerase reactivation and telomere stabilization (37). Extracts prepared from cultures of primary HUVEC, E6 HUVEC (p-15), E7 HUVEC (p15) and E6-E7 HUVEC clone 4–5–2G (p-25) were assayed for telomerase by the TRAP procedure. Telomerase was detected in E6-E7 HUVEC clone 4–5–2G line. However, using extracts containing the same amount of protein, telomerase was not detected in primary HUVEC, E6 HUVEC or E7 HUVEC (Figure 1A). In addition, TRF length analysis of E6-E7 immortalized HUVEC clone 4–5–2G (p-25) line showed that E6-E7 did not restore TRF length to precrisis level in HUVEC cells (Figure 1B).

Characteristics of HPV-16 E6-E7 immortalized line
The HPV-16 E6-E7 HUVECs exhibited the same ‘cobbledstone’ morphology (Figure 2A) as observed in normal HUVEC and developed a capillary-like tube structure upon reaching confluence (Figure 2B). The cell lines expressed Factor VIII-related antigen (Figure 2C) and took up Dil-Acetylated LDL (Figure 2D) as standard markers of endothelial origin. From the time of infection, the HPV-16 E6-E7 HUVEC immediately manifested a much increased growth rate, with a requirement for passage once every 3 days versus once a week for the control, normal cells.

Expression of viral DNA and protein
Southern blot analysis detected the presence of the HPV-16 E6 or E7 DNA in the retrovirally infected HUVECs at the 15th passage (data not shown). The control HUVECs did not contain HPV-16 E6 or E7 sequences. In addition, immunoprecipitation at 15th passage confirmed the production of the E6 and E7 proteins in both the HPV-16 E6-E7 HUVEC lines (Figure 3). Interestingly, E6 or E7 proteins were also detected in the pre-crisis E6 and E7 HUVEC cells, respectively (Figure 3).

Chromosome analysis
Evidence for the human origin of the HPV-16 E6-E7 4–5–2G line was obtained by isoenzyme analysis and cell membrane species-specific immunofluorescence. At passage 19, the E6-E7 HUVEC 4–5–2G line was aneuploid with most chromosome counts in the diploid range, and the cell line exhibited one marker chromosome. M9 = t(3qter@3p24::?3q21>qter) (Figure 4), translocation of chromosome 3, which is most commonly involved in structural alteration of HPV-immortalized human epithelial cells (50).

Tumorigenicity
When we analyzed for tumorigenic properties, we observed that none of the control and the immortalized line produced tumors in 129J nude mice even when 10^7 cells were injected. (Table I).

Expression of integrin subunits on primary and HPV-16 E6-E7 HUVEC
The integrin subunits αv, α2, α3, α6, β1, β3, and β5 have been detected on endothelial cells during angiogenesis in vivo (18–24). The expression of these integrin subunits by HUVEC (passage 3) and HPV E6-E7 HUVEC 4–5–2G (passages 10 and 25) during exponential growth was analyzed by immunofluorescence staining using integrin subunit-specific monoclonal antibodies and quantitation by flow cytometry (Figure 5). As expected, proliferating primary HUVEC cells from passage 3 exhibited strong immunofluorescence with integrin-specific antibodies for the αv3 and αvβ5 heterodimers, as well as α2, α3, α6, β1 and β4 subunits. Thus, HPV-16 E6-E7 4–5–2G cell lines maintained for 10 and 25 passages showed profiles of the integrin repertoire which was similar to that expressed by HUVECS.

The mean immunofluorescence intensity of the antibody staining for the population of each cell passage was compared
Fig. 2. Immortalized HPV-16 E6-E7 HUVEC 4–5–2G line. (A) Morphology of 4–5–2G HUVEC line. (B) Capillary-like tube structure. (C) Factor VIII-related antigen, strong granular cytoplasmic staining, especially in the perinuclear region (red-colored). (D) Dil-Ac-LDL uptake, intense uniform cytoplasmic staining in 100% of cells.

Neoplastic transformation of HPV-16 E6-E7 4–5–2G cells by Ki-ras oncogene

The lack of tumorigenicity of the 4–5–2G line led us to inquire whether its growth properties might be further altered by the introduction of an activated Ki-ras oncogene. Infection of the HPV-16 E6-E7 4–5–2G line at passage with Kirsten murine sarcoma virus (Ki-MSV) pseudotyped with baboon endogenous virus to facilitate entry into human cells, resulted in a marked alteration in cell morphology. Two to 3 weeks after infection, the cells began to pile up in focal areas (Figure 7B). The absence of any detectable alteration induced by helper virus alone implied that Ki-MSV was responsible for the induction of any detectable transformed morphology. The Ki-MSV altered cells expressed the p21 ras protein at high level, as shown by immunoprecipitation assay (Figure 7), confirming the presence and expression of the Ki-ras oncogene. In addition, telomerase was detected in E6-E7 HUVEC clone 4–5–2G as described (Figure 1, lane 5) and also in a derivative that had been transformed by the v-Ki-ras (Figure 1, lane 6). In an invasion chamber assay, the migration of the Ki-ras transformed 4–5–2G cells was ten times higher than that of the untransformed 4–5–2G cells, indicating that Ki-ras transformation is associated with the acquisition of invasion capability by the Ki-ras transformed 4–5–2G cells (Figure 7 and Table I).

When adult 129J nude mice were inoculated s.c. with 10^7 Ki-ras transformed 4–5–2G cells, tumors developed within 2–3 weeks at the site of inoculation and progressed to 25 mm in size by 5 weeks (Table I). The tumors are highly vascular and invasive. Microscopic examination led to a pathologic diagnosis of sarcoma. Focal areas of necrosis were present. The tumor was composed of closely arranged markedly atypical spindle and giant cells. The tumor cells possessed hyperchromatic nuclei which were oval to fusiform in shape, with irregular contours. Greater than 20 mitosis per 10 high power fields were seen. In some areas tumor cells appeared to forming vascular spaces (Figure 7C).

Discussion

We have described the establishment of permanent human endothelial cells which retain endothelial cell-specific characteristics. HPV-16 E6 and E7 alone failed to immortalize HUVECs even though the genes were able to extend the lifespan of HUVECs. However, the HPV-16 E6-E7 efficiently immortalize HUVECs. The parent HUVECs could not be maintained beyond 10 passages; however, the immortalized cells had 3 times the lifespan of the control and have been in continuous culture for >1 year. Telomerase activity was detected in the immortalized cells. Stable integration and expression of the HPV-16 E6-E7 DNA sequences was observed. The cells exhibited the ‘cobblestone’ morphology characteristic of HUVECs and developed a capillary-like tube...
Human vascular endothelial cell line

Fig. 4. Karyotype of HPV-16 E6-E7 HUVEC 4–5–2G cells (passage 19). Translocation of chromosome 8 was observed.

| Table I. Biological properties of the E6-E7 immortalized HUVEC cell line transformed by Ki-MSV |
|-----------------------------------------------|-----------------|
| | Uninfected | Ki-MSV infected |
| Morphology | Flat | Transformed |
| Activated K-ras expression | − | + |
| Invasion capability | − | + |
| Tumorigenicity | 0/4 | 4/4a |

Nude mice with tumors/mice inoculatedb.

aTumors were reestablished in tissue culture and confirmed to resemble the cells of origin by karyological analysis.

bNude mice were inoculated with 10⁷ cells.

structure upon reaching confluence. The line expressed Factor VIII-related antigen and took up Dil-Ac-LDL as markers of endothelial origin. The cells were not tumorigenic in athymic nude mice. The line expressed integrin subunits (αvβ3, αvβ5, β1, α2, α3, β4 and α6) consistent with an endothelial origin. The high passage of the immortalized line showed similar integrin immunofluorescence intensities to that of primary HUVECS.

Human endothelial cells, like other normal diploid human cells, have a limited lifespan, and spontaneously become senescent. The establishment of immortalized cell lines, where cells retain the differentiated phenotype and have unlimited lifespan, has been seen as an attractive alternative to the short-term use of primary cultures, and attempts have been made to produce human endothelial cell lines that retain the characteristics of a normal cell. Wild type SV40 or the SV40 T-antigen gene have been used to immortalize human endothelial cells in almost all studies (26–30). In almost all cases, either the endothelial-specific production of Factor VIII related antigen is lost or morphology of the cells deviated from primary endothelial cells even though other endothelial cell markers such as angiotension-converting enzyme are retained.

It has been documented that integration of DNA encoding the HPV-16 E6-E7 into the genome of cultured primary human cells of different origin leads to a high-efficiency of immortalization (31,51–55). The mechanism underlying this process is not fully understood, although it appears to involve an interaction between the E6-E7 and the cellular tumor suppression protein p53 and the retinoblastoma gene product. These interactions would be expected to result in the disturbance of the normal cell cycle control, leading to the immortalization (56). Recently, the successful immortalization of human endothelial cells by the HPV-16 E6-E7 and the maintenance of endothelial cell-specific properties such as Factor VIII related antigen, surface-bound antigens (endoglin, PCAM-1) and surface adhesion molecules E-selection in the immortalized
Fig. 5. Flow cytometric profile of integrin subunit expression in different HUVEC cell lines before and after transformation. Integrin expression was quantitated in HUVEC passage 3, HUVEC 4–5–2G passage 10 and HUVEC 4–5–2G passage 25 cells undergoing exponential growth as described in Materials and methods. Cells were immunostained with a murine or rat isotype control, or the integrin subunit specific antibodies: \( \alpha v \beta 3 \) LM690; \( \alpha v \beta 5 \) P1F6; \( \beta 1 \) PAC10; \( \alpha 2 \) p1E6; \( \alpha 3 \) P1B5; \( \beta 4 \) UM-A9 and \( \alpha 6 \) GoH3 followed by the appropriate PE or FITC coupled anti-Ig as described in Materials and methods. The flow cytometric profile of rat IgG isotype control was also performed in each experiment and showed a similar mean immunofluorescence intensity and distribution to that of the mouse IgG isotype control (data not shown).

Fig. 6. Mean fold-increase in fluorescence intensity of immunostaining for integrin subunits in different HUVEC cell lines. HUVECs were immunostained with antibodies as described in Figure 4, and results are presented as the fold-increase of immunofluorescence intensity, calculated by dividing mean fluorescence intensity of immunostaining for integrin subunits with that of the isotype control. The HUVEC immortalized cell lines 4–5–2G at passage 10 and 25 showed similar immunofluorescence intensities to that of the HUVEC parental cells at passage 3.

Since the HPV-16 E6 and E7 alone are known to be sufficient to immortalize certain human cells (31–35), we chose these DNA viral genes as well as E6-E7 gene to attempt to immortalize HUVECS. As described above, we have successfully introduced HPV-16 E6, E7 and E6-E7 into HUVECS. Both E6 and E7 cultures exhibited an extended lifespan but eventually underwent senescence. E6-E7 cultures, however, acquired immortality. It should be noted that E6 and E7 proteins were expressed, as evidenced by immunoprecipitation. This observation indicates that the mere presence of these proteins alone does not necessarily lead to immortalization of human endothelial cells. Additional genetic alterations are clearly required for the immortalization in E6 and E7 transformed cells. Human keratinocytes were immortalized by HPV-16 E7 but attempts to immortalize with E6 were not successful (32,33). HPV-16 E6 alone was sufficient to immortalize breast epithelium (34,35). Successful immortalization of human uroepithelial cells by HPV-16 E6 or E7 alone was recently reported (31). There are many possibilities for the apparent cell type specificity. Our data indicate that both E6 and E7 are required for immortalization of human endothelial cells.

A key factor other than p53, Rb and cellular oncogenes in the establishment of immortalized cells may be the reactivation of a telomerase (37). An important factor in the genetic instability associated with immortalization of cells may be the stability of telomere sequences. These sequences, TTAGGG repeats, located at the end of chromosomes (57) may play an important role in cell senescence. There is shortening of telomeres as a function of aging of cells and as cells are passaged in vitro. The telomeres of normal and precisis of HPV-16 DNA immortalized foreskin and cervical epithelial cells are shortened until the cells senesced or reached crisis. HPV-immortalized cells tend to restore their telomeres with passaging in culture, consistent with the hypothesis that longer telomeres are favored for cell growth in vitro (58). It has been reported that while TRF lengths in E6-E7-expressing anogenital epithelial cells undergo shortening when the cells go through crisis, TRF lengths are restored upon further culture (58). These workers have also found that in human cervical keratinocytes and mammary epithelial cells that HPV-16 E6 activates telomerase (59). In contrast, we find that for HUVEC, HPV-16 E6 does not activate telomerase (see Figure 1, lane 3). Furthermore, while E6-E7 were able to immortalize HUVEC, they did not lead to restoration of telomere lengths.
with Ki-MSV transformed 4–5–2G line; (B) and v-Ki-HUVECs in culture by a combination of HPV-16 E6-E7 immortalized cells. The significance of the combined action of HPV-16 and the v-Ki-ras oncogene in the induction of malignant transformation of HUVECs is emphasized by the apparent inability of the v-Ki-ras gene alone to induce malignant transformation unless the cells have been first immortalized by HPV-16 (data not shown). Thus, the v-Ki-ras oncogene has the ability to complement with HPV-16 to induce full transformation. Our results show that successive changes are required to induce malignant transformation of HUVECs.

We have shown for the first time the pro-angiogenic effect of Ki-ras oncogene, one of the most common dominantly acting oncogenes detected in human cancers. The invasion chamber assay demonstrated the acquisition of high invasion capability of the Ki-ras transformed HUVECs and this was consistent with formation of an invasive tumor in nude mice. It has been reported that the expression of mutant ras oncogenes is associated with marked up-regulation of vascular endothelial growth factor/vascular permeability factor in transformed rat epithelial cells and can lead to stimulation of angiogenesis (60).

Recently, in vitro studies of the role of growth and coagulation factor molecule expression in endothelium have provided the foundation for development of in vivo pharmacologic and gene therapy for cardiovascular thrombosis and cancer angiogenesis (1–3). Our unique multistep in vitro model should prove invaluable for studies of the molecular mechanisms underlying normal and neoplastic endothelial cell proliferation and migration, and prove a useful in vitro model for development of pharmacologic and gene therapy for cardiovascular thrombosis and cancer.

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References


Our data thus indicate that the involvement of oncoproteins, particularly HPV-16 E6 and E7, in telomerase activation/telomere maintenance cannot be generalized and varies among different cell types.

Most HPV-immortalized human epithelial cells are usually associated with aneuploidy and structural rearrangement of chromosomes. Chromosomes 3 and 1 are most commonly involved in structural aberrations (50). We have also observed chromosome 3 translocation in our E6–E7 immortalized cells. An increase in the number of certain chromosomes may also be critical for immortalization because the increase in damage of some genes could confer the selective advantage to the immortalized cells.

Our results demonstrate malignant transformation of HUVECs in culture by a combination of HPV-16 E6–E7 and v-Ki-ras and support a multistep process for neoplastic conversion. We have demonstrated that expression of the v-Ki-ras gene in an HPV-16 E6–E7 immortalized HUVEC line facilitated malignant transformation. Two or more alterations in cellular growth properties seem to be required. The process was initiated by the acquisition of an unlimited growth potential as a result of HPV-16 E6–E7 gene expression. Promotion to a malignant phenotype occurred with infection of Ki-MSV, a virus with an activated Ki-ras oncogene, which is known to be activated in spontaneous human tumors. Morphological alterations, invasion capability and the ability to form rapidly a growing sarcoma in athymic nude mice appeared to be concomitantly acquired properties of the v-Ki-ras transformed cells.

The process of telomere maintenance cannot be generalized and varies among different cell types.


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