Kaposi's sarcoma associated herpesvirus G protein-coupled receptor immortalizes human endothelial cells by activation of the VEGF receptor-2/ KDR

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Summary

The G protein-coupled receptor oncogene (*vGPCR*) of the Kaposi's sarcoma (KS) associated herpesvirus (KSHV), an oncovirus implicated in angioproliferative neoplasms, induces angiogenesis by VEGF secretion. Accordingly, we found that expression of *vGPCR* in human umbilical vein endothelial cells (HUVEC) leads to immortalization with constitutive VEGF receptor-2/ KDR expression and activation. *vGPCR* immortalization was associated with anti-senescence mediated by alternative lengthening of telomeres and an anti-apoptotic response mediated by *vGPCR* constitutive signaling and KDR autocrine signaling leading to activation of the PI3K/AKT pathway. In the presence of the KS growth factor VEGF, this mechanism can sustain suppression of signaling by the immortalizing gene. We conclude that *vGPCR* can cause an oncogenic immortalizing event and recapitulate aspects of the KS angiogenic phenotype in human endothelial cells, pointing to this gene as a pathogenic determinant of KSHV.

Introduction

Kaposi's sarcoma associated herpesvirus (KSHV) or Human Herpesvirus-8 (Chang et al., 1994; Mesri et al., 1996; Renne et al., 1996) is an oncogenic γ-2 herpesvirus implicated in three AIDS associated malignancies: Kaposi's sarcoma (KS) (Chang et al., 1994), Primary effusion lymphoma (Cesarman et al., 1995), and Multicentric Castleman's disease (Soulier et al., 1995). KS is the most common cancer associated with AIDS (AIDS-KS) (Antman and Chang, 2000; Gallo, 1998; Safai et al., 1985); and in its advanced disseminated form, it is still difficult to treat, causing considerable morbidity and mortality. KS lesions can be found in the skin, lymph nodes, and GI tract and typically present intense angiogenesis, proliferation of spindle-like cells of endothelial origin, and inflammatory cell infiltration (Ensoli and Sturzl, 1998; Gallo, 1998; Safai et al., 1985). Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) plays a pivotal role in the pathogenesis of KS since it is an angiogenic and KS cell growth factor ubiquitously found in the lesions (Cornali et al., 1996; Masood et al., 1997), together with its receptor-2 (VEGF-R2), also called *k*inase insert *d*omain-containing *r*eceptor (KDR) (Brown et al., 1996). However, the triggering mechanisms for the VEGF-mediated angiogenic response in KS are still a matter of controversy (Gallo, 1998).

Cumulative evidence shows that KSHV fulfills all of the Koch-like postulates (Zur Hausen, 1999), defining it as an "angiogenic oncovirus" necessary for KS (Boshoff and Weiss, 1998; Ganem, 1998b): (1) KSHV is consistently found in the lesions of all clinical

SIGNIFICANCE

KSHV angiogenic oncogenes can cause endothelial cell transformation leading to Kaposi's sarcoma (KS), an angioproliferative disorder characterized by proliferation of spindle cells of endothelial origin expressing VEGF and its receptor-2/ KDR. However, since these are lytic genes not expressed in most of KSHV-infected KS spindle cells, their role in oncogenesis has been unclear. Our observation that *vGPCR* immortalizes HUVEC by autocrine KDR activation demonstrates the oncogenicity of KSHV lytic genes in human endothelium since it shows that *vGPCR* expression is pro-angiogenic and pre-neoplastic, pointing to *vGPCR* and KDR activation as targets for KS therapy. Evidence indicating that shutdown of *vGPCR* signaling can be overcome by VEGF, a KS growth factor, provides a molecular explanation for the feasibility of a "hit and run" oncogenic event initiated by *vGPCR* in the multistep endothelial oncogenesis process leading to KS.

forms of KS (Chang et al., 1994; Moore and Chang, 1995); (2) KSHV infection precedes KS development and overlaps with KS risks (Kedes et al., 1996; Moore et al., 1996b; Whitby et al., 1995); (3) KSHV is found in KS-spindle and endothelial cells in the lesions (Boshoff et al., 1995); (4) KSHV infection of endothelial cells, the cells thought to be pre-malignant for KS spindle cells, was found to cause spindle cell transformation (Ciufo et al., 2001; Lagunoff et al., 2002; Moses et al., 1999) and, in some cases, immortalization (Flore et al., 1998) and angiogenic alterations such as upregulation of KDR (Flore et al., 1998; Moses et al., 2002); (5) The KSHV genome (Russo et al., 1996) contains genes with the potential to induce cell transformation (Bais et al., 1998; Friborg et al., 1999; Gao et al., 1997; Godden-Kent et al., 1997; Lee et al., 1998; Muralidhar et al., 1998; Radkov et al., 2000), immune deregulation (Moore et al., 1996a; Nicholas et al., 1997), and angiogenicity (Aoki et al., 1999; Bais et al., 1998; Boshoff et al., 1997; Yang et al., 2000). Nevertheless, KSHV can cause KS only in certain settings. According to the serological data, about 5% of the general population may be infected with KSHV; however, the incidence of KS is below 1 in 100,000 in the general population. KS incidence increases almost 20,000 times in AIDS, suggesting that immune deregulation and HIV are important co-factors for KS- and KSHV-mediated pathogenesis (Antman and Chang, 2000; Gallo, 1998). KSHV pathogenicity will be determined by the specific pattern of viral gene expression and the specific cellular background in which these genes are expressed, both of which are highly dependent on host factors. Like other herpesviruses, KSHV can lead either to a latent infection or to a productive or lytic infection that destroys the infected cell (Ganem, 1998b). The great majority of the cells in the KS lesions are latently infected with KSHV (Staskus et al., 1997). Paradoxically, many of the KSHV genes found to be transforming or pathogenic are lytic genes, which are only expressed by a subset of cells in KS lesions productively infected with KSHV (Staskus et al., 1997; Sun et al., 1999). This observation is consistent with a role of latent genes in maintenance of the malignant phenotype and a role for lytic genes expressed by productively infected cells in paracrine angiogenesis and the initiation of the phenotype (Ganem, 1998b).

Canonical latent and lytic KSHV infections are not highly oncogenic as suggested by the low incidence of KS in the general KSHV-seropositive population, the low efficiency of EC transformation in vitro (Flore et al., 1998), and the inability of KSHV latent infection per se to transform endothelial cells (Lagunoff et al., 2002). Thus, a critical question becomes how the interplay between KSHV and host gene expression leads to endothelial cell transformation and establishment of the KS angio-proliferative lesion. Therefore, we focused on angiogenic lytic genes because their expression in endothelial cells could lead to oncogenic alterations. KSHV-ORF74 is an early lytic gene encoding a G protein-coupled receptor (KSHV-GPCR/*vGPCR*) that shares a high degree of homology with the human CXCR2 and CXCR1 (Cesarman et al., 1996), the receptors for the angiogenic chemokines IL-8 and Gro-α, and can signal both in a constitutive (ligand-independent) (Arvanitakis et al., 1997) and chemokine-activated manner (Gershengorn et al., 1998). Expression of *vGPCR* is sufficient to induce cell transformation and VEGF-mediated angiogenesis in NIH3T3 cells (Bais et al., 1998); and in transgenic animals expressing *vGPCR* in cells of the T cell lineage, to induce VEGF-driven KS-like lesions (Cesarman et al., 2000; Yang et al., 2000). Expression of *vGPCR* in endothelial cells has been shown to activate survival cascades, to modulate the expression of angiogenesis regulating genes and cytokines (Montaner et al., 2001; Pati et al., 2001; Polson et al., 2002), and to activate pro-inflammatory transcription (Chiou et al., 2002; Pati et al., 2001). These results and its pattern of expression in KSHV-infected endothelial-lineage cells of KS lesions (Chiou et al., 2002; Kirshner et al., 1999; Staskus et al., 1997) indicate that *vGPCR* could participate in KS pathogenesis. In the present study, we found that expression of *vGPCR* is sufficient to cause endothelial cell immortalization with constitutive expression and autocrine activation of VEGF receptor-2/KDR, a biological response related to the ability of KSHV to induce endothelial oncogenesis and angiogenesis in KS.

Results

Expression of *vGPCR* in primary endothelial cells causes immortalization

To test the effect of sustained *vGPCR* expression in endothelium, we transduced HUVEC cells at low passage with a neomycin-resistant retrovirus encoding an epitope-tagged version of *vGPCR* (*HA-vGPCR*) using an *EGFP*-transducing virus as control. After a single round of neomycin selection, we found that 100% of the cells expressed the transgene as determined by UV fluorescent microscopy (*EGFP*) or anti-HA immunostaining (Figures 1A and 1B). *vGPCR*-expressing cells showed strikingly altered morphology. Both sub-confluent and confluent vGPCR-EC were spindle shaped and refractile (Figure 1C). Confluent cultures showed loss of both cell junctions and the typical cobblestone appearance (Figure 1C). vGPCR-EC also developed characteristic cell extensions such as lamelipodia and pseudopodia best shown by confocal microscopy (Figure 1B). The most striking consequence of *vGPCR* gene transduction was that the vGPCR-EC were immortal in culture (Figure 1D). To date, they have been passaged every week for 2 years, maintaining the same morphologic appearance and behavior in culture. In contrast, and as expected for primary EC, both EGFP-EC and non-transduced HUVEC became senescent, flattened, and went into terminal replicative arrest between passages 12 and 14 (Figure 1D) . A Southern blot of the vGPCR-EC hybridized with a *vGPCR* probe (data not shown) to detect the provirus showed that the population by passage 20 was oligoclonal, with two major and three minor bands, indicating that immortalization was not the consequence of a unique retroviral insertion event. Although the vGPCR-EC are immortal, they do not have all characteristics of transformed cells since they cannot be cloned and they are not tumorigenic in immunodeficient mice (not shown). Furthermore, in culture medium, these cells divided at a rate similar to low passage HUVEC and had similar thymidine uptake levels in serum free conditions (15,000 $\pm$ 300 versus 12,500 $\pm$ 250 cpm/24 hr). These data are consistent with a multistep oncogenic process, with *vGPCR* expression providing the immortalizing step.

vGPCR immortalization of HUVEC occurs along with expression of the VEGF receptors

To determine if *vGPCR* immortalization was associated with high levels of VEGF receptor-2/KDR expression as seen in KSHV infected endothelial cells in vitro and in KS lesions, we carried out flow-cytometric analysis of vGPCR-EC and HUVEC at multi-

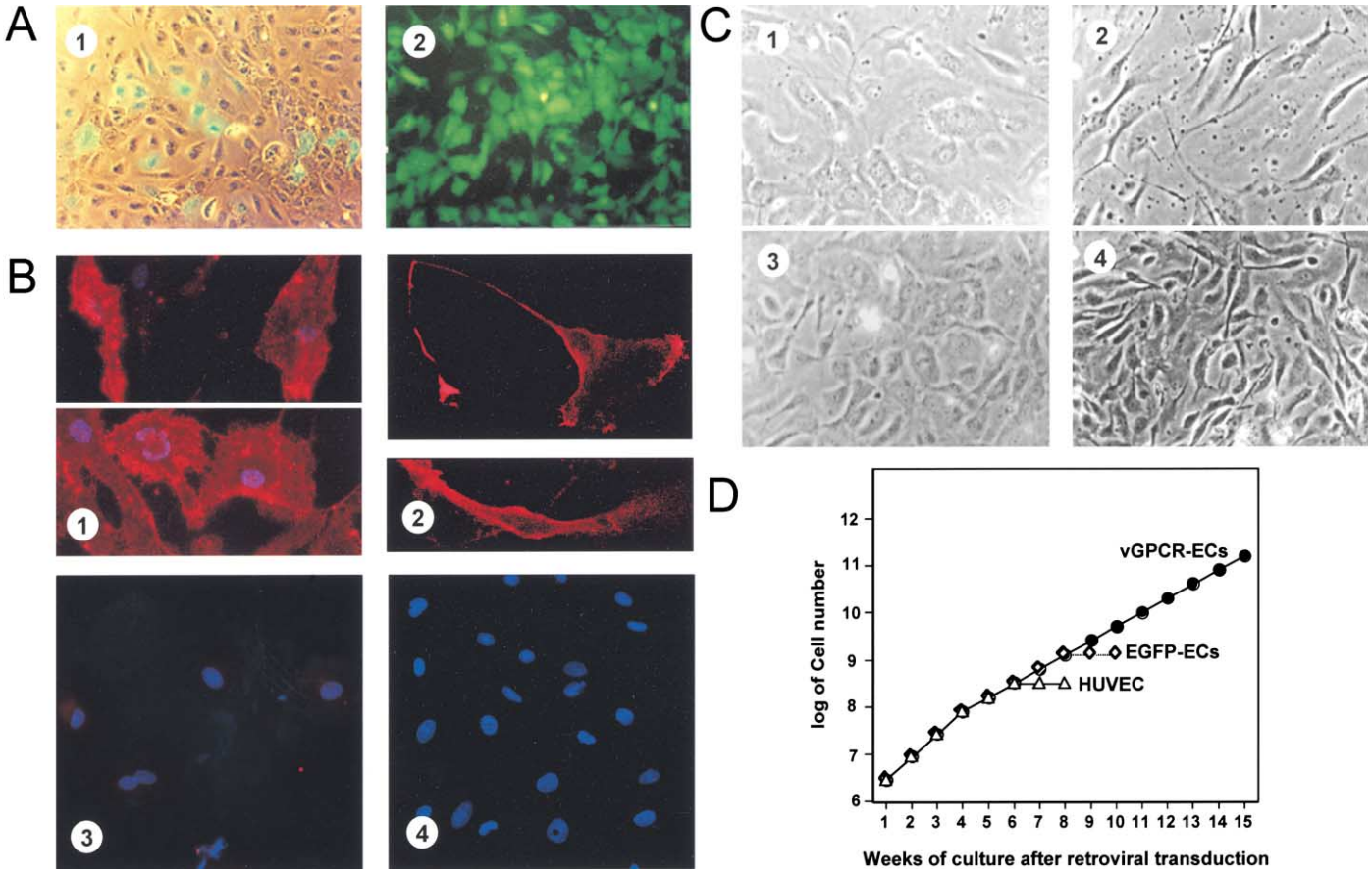

Figure 1. Retroviral transduction of *HA-vGPCR* to HUVEC leads to a spindle-cell phenotype and cell immortalization

A: (1) Phase-contrast microscopy of confluent cultures of HUVEC transduced with *EGFP* retrovirus after initial transduction. (2) UV microscopy of *EGFP*-transduced HUVEC after a round of G418 selection.

B: Anti-HA immunohistochemistry of *vGPCR*-transduced HUVEC selected with G418 (1 and 2) and EC and IBMEC controls (3 and 4) showing the expression of *HA-vGPCR* on the surface of the cells. For panels 1, 3, and 4, immunostaining developed with Vector red and cell nuclei stained with DAPI were visualized by UV fluorescent microscopy. The panel 2 shows anti-HA immnostained vGPCR-EC cells single-developed with Vector Red and visualized by UV confocal microscopy through a Z plane passing through the cell nucleus.

C: Phase contrast microscopy of sub-confluent (1 and 2) or confluent (3 and 4) cultures of EGFP-EC (Panels 1 and 3) or vGPCR-EC (Panels 2 and 4).

D: Logaritmic plot of cell growth. After neomycin selection (week 1), *vGPCR*-transduced cells, *EGFP*-transduced cells, or control HUVEC were passed at 1:3 (weeks 1–4) or 1:2 (from week five) weekly splitting ratio. The graph shows up to week 15 after G418 (current number of passage for vGPCR-EC is 160). Similar data were obtained with an independently generated culture from the same viral batch and with different viral batches generated at a later date.

ple passages using a high-affinity anti-KDR monoclonal antibody (Clone 6.64) (Peichev et al., 2000). Figure 2A shows that, as expected, low passage HUVEC displayed high levels of KDR expression in the majority of the cells. vGPCR-EC at passage 12, a passage at which control EGFP-EC started to senesce, showed high levels of KDR expression in 95% of the population (Mean Fluorescence 1 × 10 E 7.48) (Figure 2A); this was maintained in immortalized cultures of vGPCR-EC (passage 40), which also showed high levels of expression of KDR (Mean Fluorescence 1 × 10 E 8.45) in the majority of the cells (91%). Immunohistochemical studies shown in Figures 2B and 2C confirmed the flow cytometry results for KDR and also showed that *vGPCR* cells also expressed high levels of the VEGF-R1/flt-1 (Figure 2C). In addition to VEGF receptors, vGPCR-EC also expressed the endothelial cell markers CD31/PECAM as determined by FACS and Tie-2 as determined by RT-PCR (not shown). Expression of VEGF-R2/KDR and VEGF-R1/flt-1 is not a general feature of immortalized EC. SV40 Large T antigen-immortalized endothelial cells (IBMEC) completely lacked expression of KDR and VEGF-R1. There is only one other known example of a host-restricted endothelial viral oncogene that immortalizes with maintenance of KDR expression: it is the *Polyoma middle T* antigen (*PymT*) that, similarly to *vGPCR*, is an endothelial cell-transforming signaling oncogene of a murine virus (Pepper et al., 1995).

vGPCR induces telomere stabilization by a telomerase-independent mechanism

Cell immortalization is the consequence of overriding the normal cellular mechanisms of senescence and apoptosis. To investigate the molecular basis of the immortalizing event induced by *vGPCR* expression, we examined telomere maintenance and telomerase activity as well as the ability of *vGPCR* and KDR to promote anti-apoptotic responses in the immortalized cells. Southern blots displayed on Figure 3 show that two independently established vGPCR-EC cultures at passage 25 had telo-

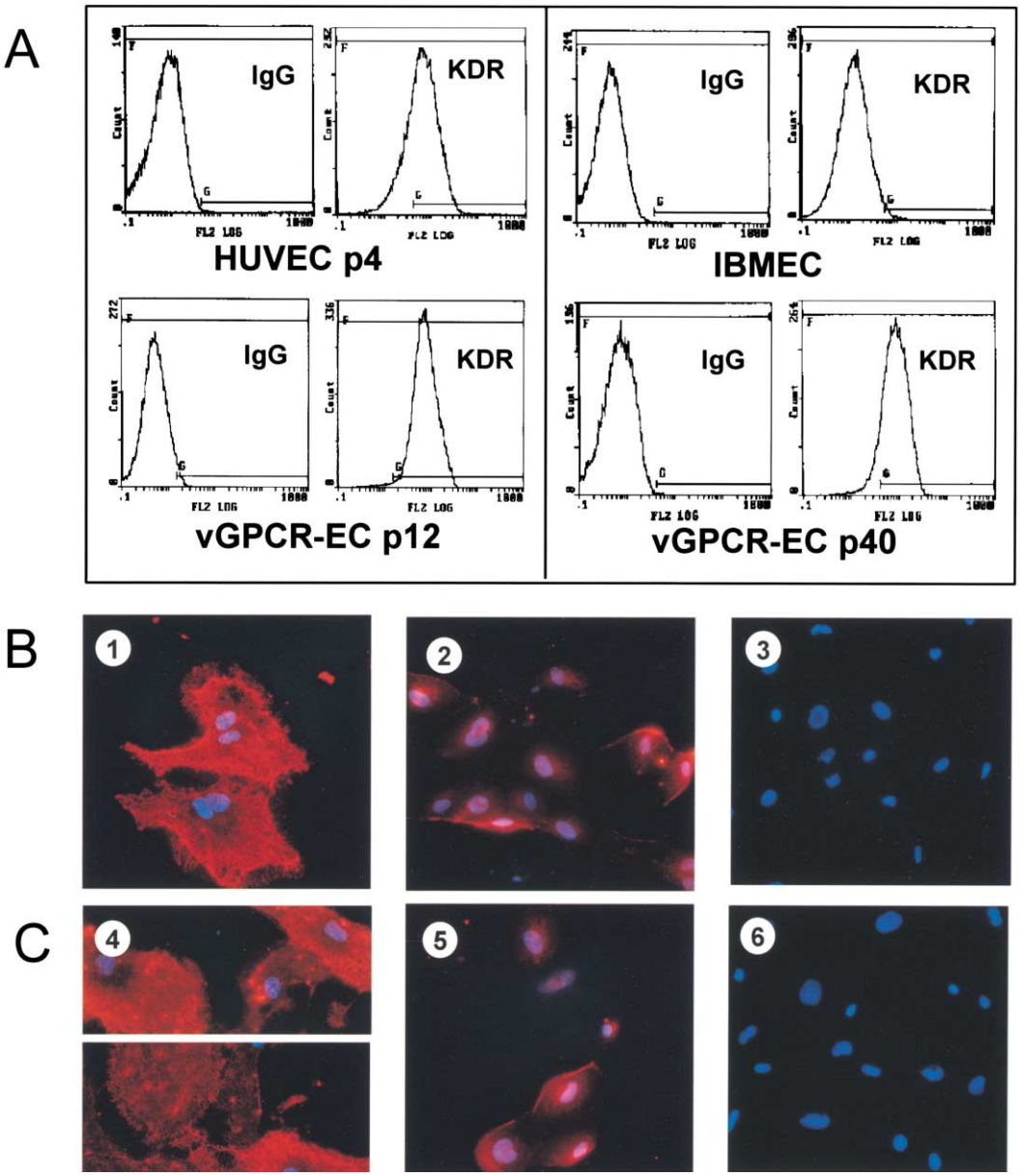

Figure 2. *vGPCR*-mediated immortalization is associated with expression of VEGF receptors

A: Flow cytometric analysis of VEGF-R2/KDR expression comparing HUVEC (passage 4), vGPCR-EC passage 12, vGPCR-EC passage 40, or IBMEC. Cells were maintained O.N. in serum free medium, harvested by collagenase digestion, and stained with control IgG or anti-KDR PE-labeled antibody (MoAb 6.64).

B and **C:** Immunohistochemical detection of VEGF-R2/KDR (**B**) and VEGF-R1/flt-1 (**C**) in vGPCR-EC (1 and 3), dermal microvascular EC (DMVEC) (positive control) (2 and 5), and IBMEC (3 and 6). Bound antibody was detected by incubation with anti-mouse Ig labeled with alkaline phosphatase, developed with Vector Red, and visualized by UV fluorescent microscopy. DAPI was used to stain cell nuclei.

mere lengths that did not differ significantly from that of passage 4 HUVEC (Figure 3A). In contrast, SV40 LT immortalized EC (IBMEC) showed significantly shortened telomeres. These results are consistent with the observation that *vGPCR* cells became immortalized without going through a noticeable "cell crisis" triggered by telomere shortening and suggest that *vGPCR* may immortalize EC by early activation of cellular mechanisms that regulate telomere length stability. In 85% of cell lines and human cancers, telomere stability depends on activation of expression of the catalytic subunit of the telomerase (TERT) (Colgin and Reddel, 1999). In the remaining 15%, telomere maintenance is achieved by a TERT-independent mechanism or ALT (alternative lengthening of telomeres) (Colgin and Reddel, 1999; Reddel, 2000; Reddel et al., 2001). To test for TERT activity in the vGPCR-EC, we carried out a *t*elomere *r*epeat *a*mplification *p*rotocol (TRAP) assay (see Experimental Procedures). As shown in Figure 3B, vGPCR-EC, unlike positive control 293T cells, did not have detectable TERT activity. An RT-PCR assay showed that 293T expressed a *TERT* transcript while *vGPCR* cells did not (Figure 3C). These data suggest that in *vGPCR* immortalized

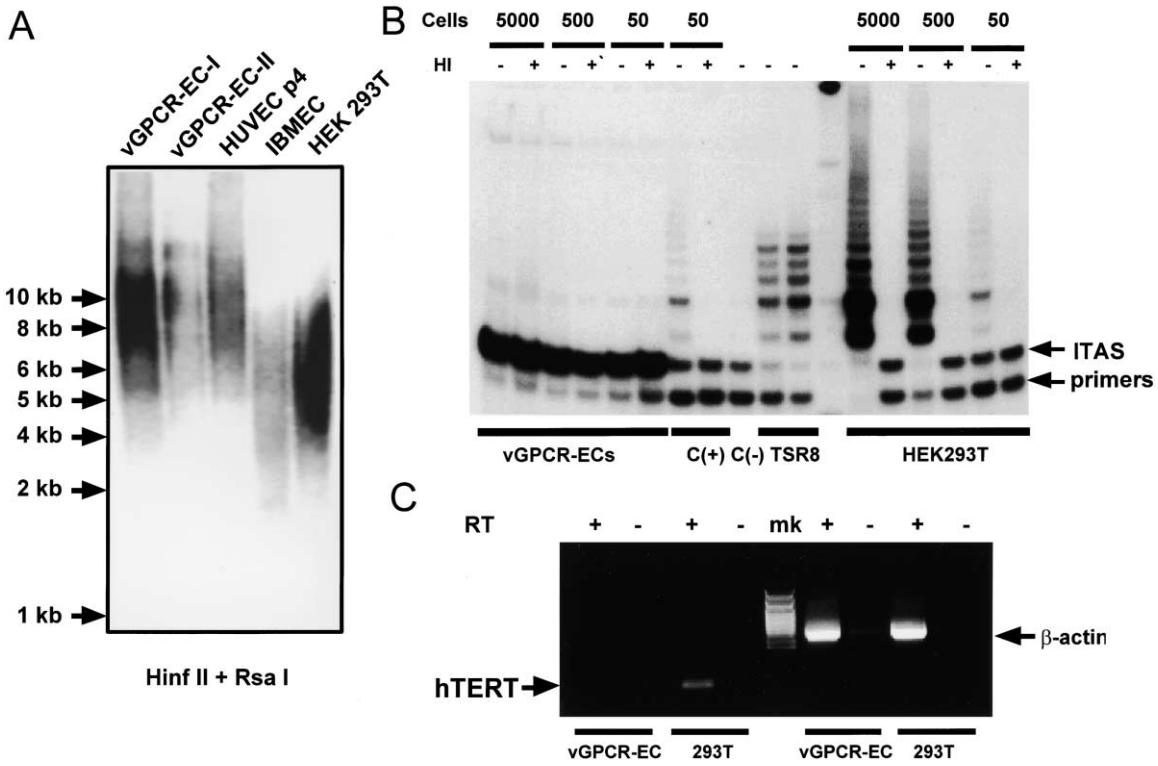

Figure 3. *vGPCR* induces telomere stabilization by a telomerase-independent mechanism

A: DNA from HUVEC passage 4, two independently derived vGPCR-EC cultures at passage 25, HEK293T cells, or IBMECs were digested with HinfII/Rsa I, separated in agarose gel, transferred to Hy-bond, hybridized with a telomeric probe, and developed by autoradiography.
B–C: TRAP assay of vGPCR-EC or HEK 293T cells (positive control); HI: heat inactivation; c(+) c(−) telomerase positive and negative lysates from the kit. ITAS: internal template amplification sequence is an internal PCR control. TSR8: synthetic telomeric control. Telomerase positive lysates generate a telomeric ladder which is amplified by PCR with labeled oligonucleotides, resolved by 12.5% PAGE, and developed by autoradiography.
C: RT-PCR for *hTERT* and β-actin of vGPCR-EC and HEK 293T (293T) mRNA.

cells, telomere maintenance is carried out by a telomerase-independent ALT mechanism (Reddel et al., 2001).

Survival of *vGPCR*-immortalized endothelial cells is mediated by *vGPCR* and KDR signaling

To determine anti-apoptotic mechanisms involved in cell immortalization, we identified signaling mechanisms involved in vGPCR-EC survival. Figures 4A and 4D show that Gro-α, a full agonist of the *vGPCR* (Gershengorn et al., 1998; Rosenkilde et al., 1999) and VEGF, the KDR ligand, rescued vGPCR-EC from starvation. As expected, VEGF was also able to rescue HUVEC while the effects of Gro-α were *vGPCR* specific. To demonstrate if these receptors play an active role in vGPCR-EC survival, we suppressed their signaling using the chemokine IP10, a *vGPCR* reverse agonist (Geras-Raaka et al., 1998; Rosenkilde et al., 1999), or PTK787, a small molecule inhibitor of the KDR tyrosine kinase (Wood et al., 2000). As shown in Figures 4B and 4C, we found that IP10 suppressed vGPCR-EC survival in serum free conditions by more than 80%, leading to more than a 2-fold increase in apoptotic cell death. Although HUVEC may express an IP10 receptor (Soejima and Rollins, 2001), IP10 did not alter the survival of control HUVEC (Figures 4B and 4C). We similarly found that PTK787 suppressed survival and increased apoptosis of vGPCR-EC (Figure 4E). Cell death and apoptosis were induced at doses up to 100-fold lower than those required to kill HUVEC (Figures 4E and 4F). These results indicate that sustained basal signaling through KDR is critical for the survival of immortal vGPCR-EC while it is not critical for the survival of primary HUVEC; this suggests that sustained high levels of KDR expression and its activation play a role in maintenance of the immortalized phenotype in vGPCR-EC. Taken together, these results show that both *vGPCR* and KDR constitutive activation induce anti-apoptotic signaling cascades mediating survival of vGPCR-EC.

Survival of immortalized vGPCR-EC is mediated by KDR and *vGPCR* activation of the PI3K/AKT pathway

Signaling by angiogenic receptors such as KDR in EC leads to the activation of the Phosphoinositide 3-OH kinase (PI3K)/AKT anti-apoptotic pathway(Gerber et al., 1998). Recent overexpression studies using transient transfection have shown that *vGPCR* could activate AKT in EC (Montaner et al., 2001). Since it is difficult to determine constitutive activation of kinases of rapid turnover such as AKT, we used the *vGPCR* ligand Gro-α and the KDR ligand VEGF to determine if *vGPCR* and KDR can activate the AKT signaling cascades in vGPCR-EC. Figure 5A shows that vGPCR-EC treatment with Gro-α or with VEGF leads to phosphorylation of AKT. AKT phosphorylation was rapid, occurring in less than 10 min, and *vGPCR* specific, as it was not detected in control HUVEC. The immediate phosphorylation

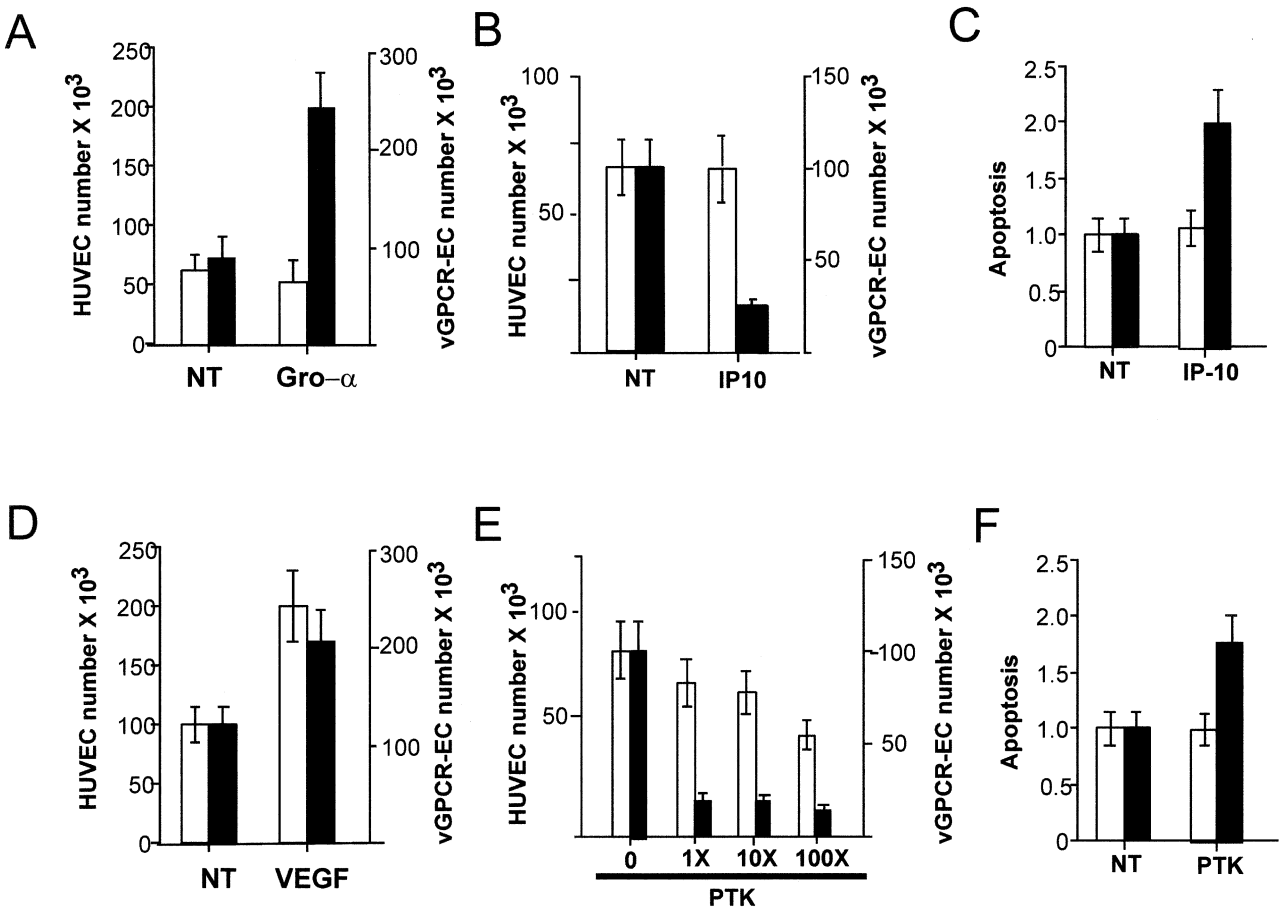

Figure 4. Survival of immortalized vGPCR-EC is mediated by KDR and *vGPCR*

A–F: Confluent cultures of HUVEC □ or vGPCR-EC ■ were incubated overnight in serum free medium, alone (No Treatment, NT) or in the presence of Gro-α (25 nM), IP10 (300 nM), VEGF (100 ng/ml), or KDR inhibitor PTK787 at 0.1 ug/ml (1×), 1 ug/ml (10×), or 10 ug/ml (100×). After 24 hr, cells were counted (**A**, **B**, **D**, and **E**) or used for measuring apoptosis (**C** and **F**) by ELISA. Changes in cell apoptosis were calculated by normalizing the values of the O.D.s to the O.D. value of the control experiment (NT). Bars indicate mean values of duplicates ± range.

of AKT by *vGPCR* activation indicates that activation of this pathway is a direct consequence of *vGPCR* signaling. To demonstrate the role of AKT signaling on survival of immortalized cells, we used the PI3K-specific inhibitor LY294002. Figure 5B shows that in serum free media, LY294002 completely suppressed vGPCR-EC survival and led to rapid apoptotic death at doses that did not affect HUVEC cell survival or apoptosis. Additionally, Figure 5C shows that the inhibitor suppressed survival of vGPCR-EC in media containing FBS and bFGF (ECM). Taken together, the signaling and survival data suggest that both *vGPCR* constitutive signaling and KDR activation mediate survival of vGPCR-EC by the PI3K/AKT pathway.

Autocrine activation of KDR by increased VEGF secretion mediates survival of vGPCR-EC

To further study the nature of the basal KDR activation mechanism implicated in cell survival, we used two types of tools, the KDR inhibitor PTK787 and a KDR-blocking antibody (IMC-1C11) (Hunt, 2001). We found that the PTK inhibitor inhibited 85% and that the anti-KDR monoclonal antibody led to 55% ($p < 0.001$) reduction of vGPCR-EC survival (Figure 6A), without significantly affecting the survival of the HUVEC. To demonstrate the molecular effectiveness of the KDR inhibitors, we determined in parallel their ability to suppress VEGF-induced tyrosine phosphorylation of the KDR receptor and to suppress VEGF activation of AKT (Figures 6B and 6C). We found that PTK completely suppressed VEGF-induced tyrosine phosphorylation of KDR while IMC-1C11 greatly decreased AKT phosphorylation mediated by VEGF (Figures 6B and 6C). Taken together, these results indicate the presence of an autocrine loop. The fact that the cell-permeable small molecule inhibitor PTK787 was much more effective than the antibody may indicate the existence of an intracellular or so-called "private" autocrine loop, such as the one recently described for endothelial hematopoietic progenitors (Gerber et al., 2002). Autocrine activation of KDR could be the consequence of VEGF overexpression caused by *vGPCR* (Bais et al., 1998). To determine whether KDR basal survival signaling was promoted by an autocrine loop, we determined VEGF levels by ELISA (Figure 6D). We found that vGPCR-EC produced ten times more VEGF than HUVEC. Taken together, these results show that *vGPCR* immortalization is partly driven by a VEGF-KDR autocrine loop induced by increased VEGF expression.

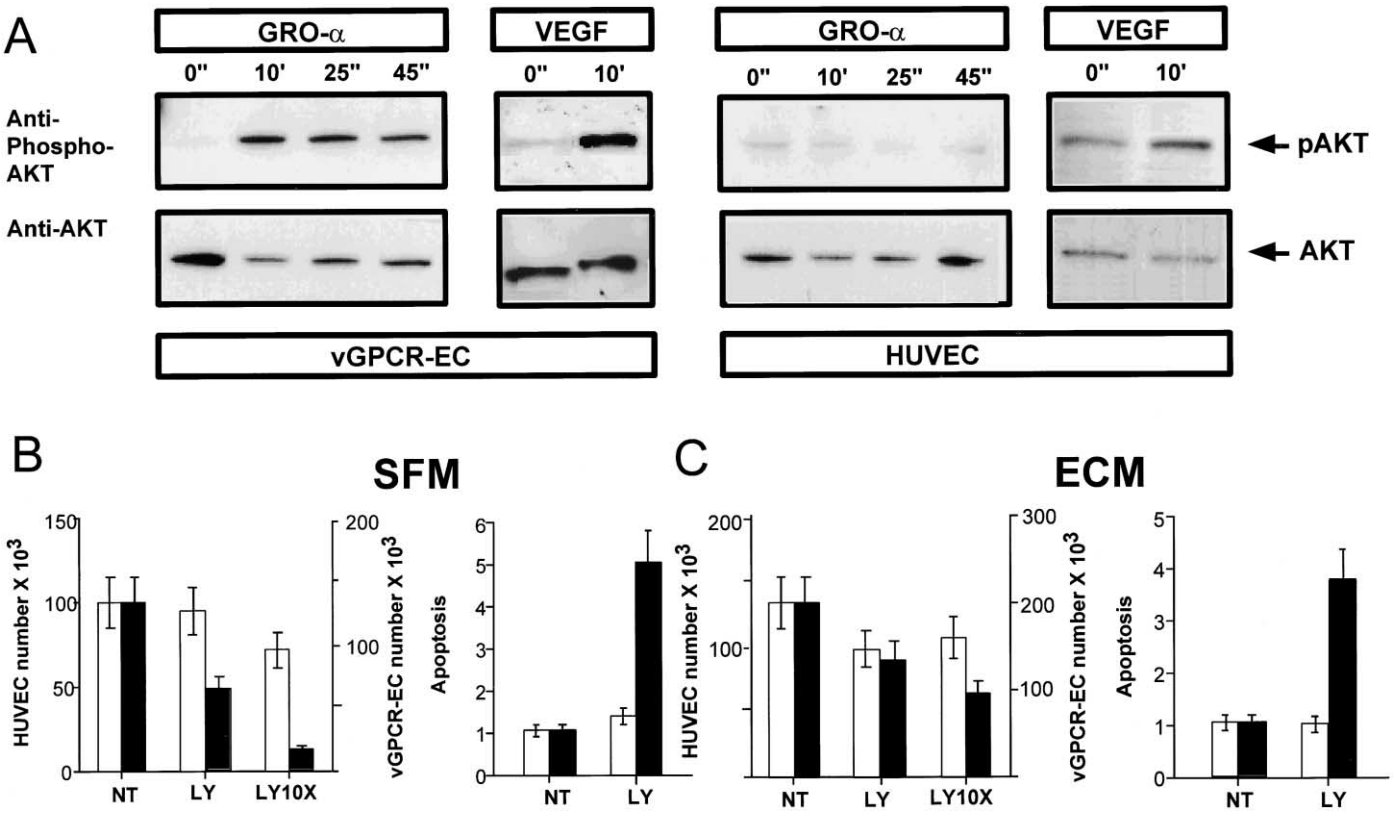

Figure 5. Survival of immortalized vGPCR-EC is mediated by KDR and *vGPCR* activation of AKT

A: Confluent cells were starved O.N. in serum free medium and stimulated or not with Gro-α (25 nM) or VEGF (100 ng/ml) for the indicated times and lysed in electrophoresis cracking buffer. Total and phospho AKT in the cell lysates were detected by Western blot using anti-phospho antibodies and antibodies to total AKT as indicated.

B–C: Confluent cultures of HUVEC □ or vGPCR-EC ■ were incubated overnight in complete endothelial cell medium (ECM) or serum free medium (SFM) as indicated in the figure, alone (No Treatment, NT) or in the presence of LY294002 (15 uM 1×, or 150 uM 10×). After 24 hr, cells were counted or used for measuring apoptosis as in Figure 4. Changes in cell apoptosis were calculated by normalizing the values of the O.D.s to the O.D. value of the control experiment (NT). Bars indicate mean values of duplicates ± range.

VEGF activation of KDR can compensate for the lack of *vGPCR* signaling in vGPCR-EC

The fact that *vGPCR* immortalization involves autocrine activation of KDR and that KDR can be further activated by paracrine addition of VEGF suggests that KDR may provide a VEGF-dependent pathway able to complement the absence of *vGPCR* survival signaling during viral latency. This could explain the observations that *vGPCR* itself is not expressed in the majority of KSHV-transformed cells of KS lesions or in KSHV-transformed endothelial cells and that KSHV-transformed cells are strictly dependent on external VEGF for survival. To test this hypothesis, we studied the effect of exogenous VEGF in the presence of IP10, which completely shuts down *vGPCR* signaling. As shown in Figure 7A, VEGF counteracted the effect of IP10, increasing cell survival above that of nontreated cells and suppressing apoptosis.

Discussion

Major unresolved issues in KS pathogenesis include the origin of the VEGF-dependent angiogenic and malignant phenotype of the lesions (Gallo, 1998) and the precise mechanistic role of KSHV infection, an absolute requirement for the development of KS (Ganem, 1998a, 1998b; Boshoff and Weiss, 1998). In this paper, we have shown that expression of the angiogenic oncoprotein of KSHV, the *vGPCR*, in human endothelial cells caused immortalization that was mediated by both constitutive *vGPCR* signaling and autocrine-KDR signaling, leading to downstream activation of the pro-survival molecule AKT. This identifies *vGPCR* as a KSHV gene that can deliver an immortalizing oncogenic hit and activate an angiogenic phenotype in target endothelial cells.

Other viral oncogenes, such as *SV40 Large T*, *HPV-16 E6/E7 Adeno E1A*, cause cell immortalization by inhibiting the activity of tumor supressor genes (Nevins and Vogt, 1996; Zur Hausen, 1999), which leads to unrestricted cell growth; this, in turn, causes telomere shortening and cell crisis that is resolved by activation of telomerase. *vGPCR* appears to belong to a category of cell-restricted signaling oncogenes, such as Polyoma *middle T* (Pepper et al., 1995) and *HTLV-1 Tax* (Yoshida, 1999), that can immortalize virus-infected cells by promotion of cell-specific anti-senescent, anti-apoptotic, and autocrine responses. Since in our studies immortalization occurred without noticeable cell crisis and telomere shortening, we conclude that *vGPCR* acts early in the oncogenesis process by activating anti-senescence and anti-apoptotic mechanisms. Our data also

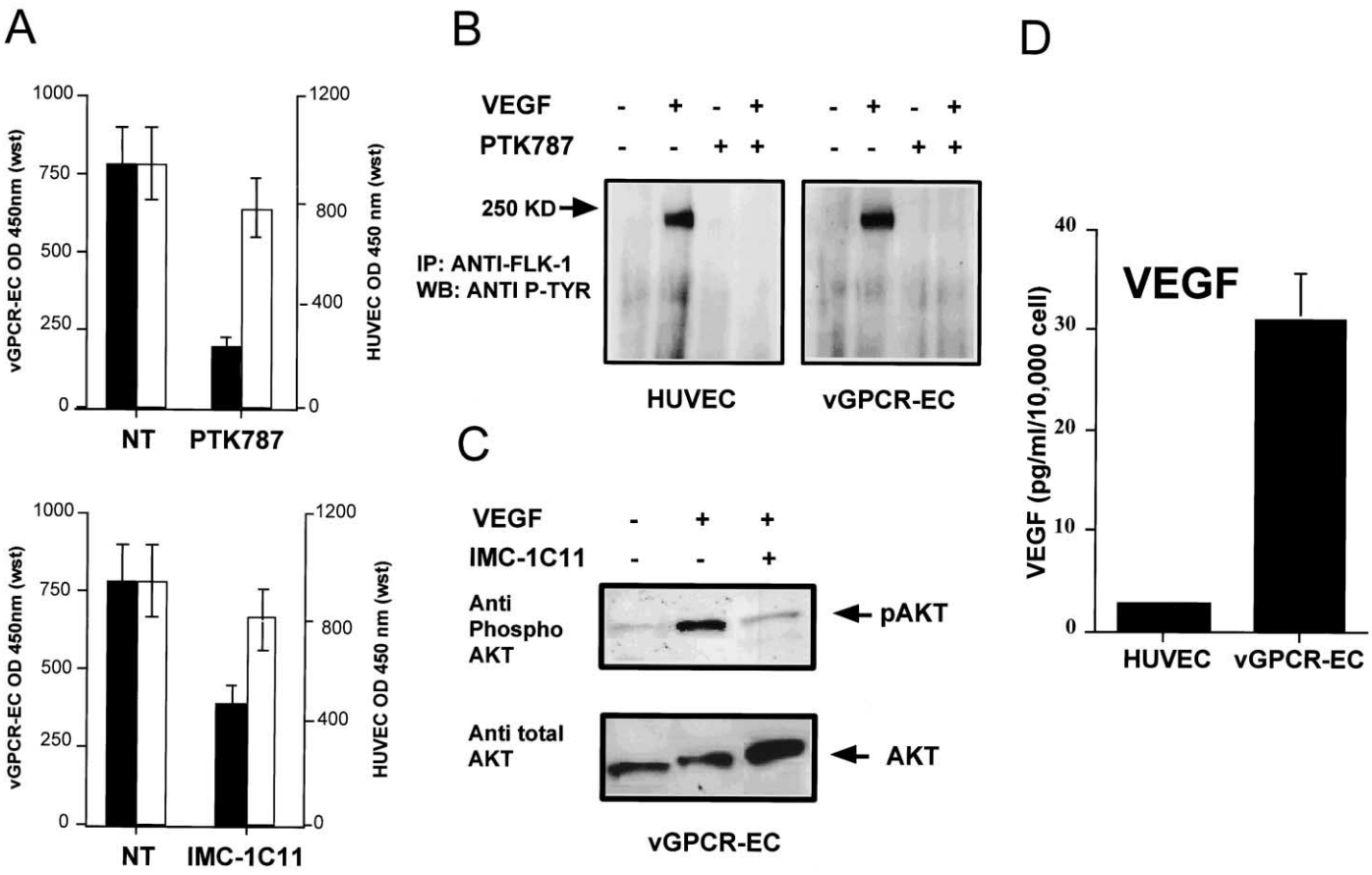

Figure 6. Autocrine activation of KDR by increased VEGF secretion mediates survival of vGPCR-EC

A: Specific inhibition of cell survival by the KDR tyrosine kinase inhibitor PTK787 (upper panel) and the anti-KDR blocking antibody IMC-1C11 (lower panel): confluent cultures of vGPCR-EC or HUVEC were incubated in serum free medium as indicated alone (No Treatment, NT) or in the presence of KDR inhibitor PTK787 at 0.1 ug/ml (1×) (upper panel), or the IMC-1C11 antibody at 2.5 ug/ml (lower panel) as indicated. After 24 hr of treatment, media were replaced by ECM containing wst-1 cell proliferation reagent and incubated at 37°C for 30 min. Formation of formazan by living cells was measured at O.D. 450 nm. Bars indicate mean values of duplicates ± range. HUVEC □, vGPCR-EC ■.

B: Confluent cultures maintained O.N. in serum free media were pretreated for 1 hr with the inhibitor prior to the addition of VEGF at 100 ng/ml for 10 min. Cells were lysed, and KDR was immunoprecipitated with an anti-flk-1 antibody, Western blotted, and developed with an anti-Phospho tyrosine monoclonal antibody as described in Experimental Procedures.

C: Confluent cultures maintained O.N. in serum free media were treated or not with VEGF at 100 ng/ml for 10 min with or without the addition of 2.5 ug/ml of IMC-1C11 antibody. Cells were lysed, and total and phospho AKT were detected by Western blot using anti-phospho antibodies and antibodies to total AKT as indicated.

D: Stable *vGPCR* expression in HUVEC leads to increased VEGF secretion: VEGF in post-culture supernatants obtained by 48 hr incubation with ECM was measured using an ELISA kit. Values were normalized to number of cells.

suggest that the endothelial-immortalizing activity of *vGPCR* is related to its ability to induce an angiogenic phenotype since we found that KDR activation, in addition to *vGPCR* expression, was necessary to mediate the survival arm of the immortalized phenotype. Moreover, since VEGF is known to be anti-senescent in EC (Watanabe et al., 1997), it is possible that EC immortalization is maintained by KDR signaling. Constitutive KDR expression in *vGPCR*-immortalized EC can be related to the existence of upregulation mechanisms. Autocrine VEGF has been shown in other systems (Weisz et al., 2001) to upregulate *KDR* expression, and we have shown with a heterologous system not expressing *KDR* that *vGPCR* can promote transcription from the *KDR* promoter in the presence of endothelial-specific transcription factors (A.V.G. and E.A.M., unpublished data).

A key aspect of the studies reported in this manuscript are the survival experiments in which vGPCR-EC are compared to HUVEC. Although HUVEC are the best "formal" control because they are the nontransduced origin of vGPCR-EC cells, they are not a perfect control because they are mortal cells with normal endothelial physiology. Studies with many small molecule inhibitors have shown that oncogenically modified cells are in a delicate "balancing act" and are more vulnerable than normal cells to inhibition of the signaling pathways that support their unlimited survival. This is also the case for vGPCR-EC, which are very sensitive to *vGPCR* inhibition and are more sensitive than HUVEC to inhibition of AKT and KDR. Their increased sensitivity to KDR inhibition coupled with their VEGF secretion strongly point to the existence of an autocrine loop. The fact that the small molecule inhibitor PTK787 is more effective than the KDR-blocking antibody could be an indication of intracellular KDR

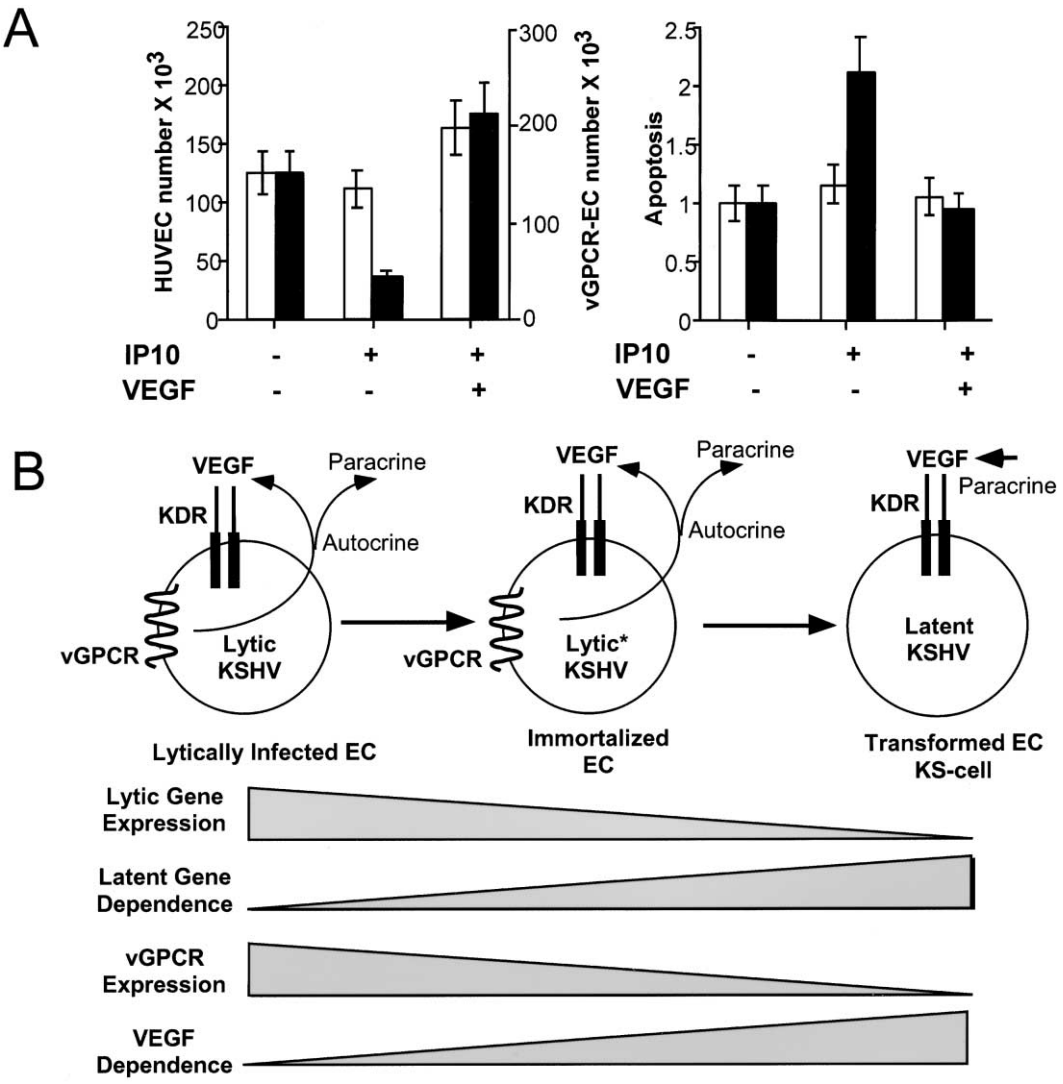

Figure 7. VEGF activation of KDR can compensate for the lack of *vGPCR* signaling in vGPCR-EC

A: Confluent cultures of vGPCR-EC or HUVEC were incubated overnight in serum free medium, alone or in the presence of IP10 (300 nM) or VEGF at 100 ng/ml. After 24 hr, cells were counted or used for measuring apoptosis as indicated. Changes in cell apoptosis were calculated by normalizing the value of the O.D.s to the O.D. value of the control experiment (no additions). Bars indicate mean values of duplicates ± range. HUVEC □, vGPCR-EC ■.

B: Model for *vGPCR* "hit and run" immortalization and paracrine activation of angiogenesis in KS pathogenesis: during lytic infection, *vGPCR* expression in EC could lead to the establishment of a VEGF–KDR loop which is oncogenic, and to increased secretion of VEGF which can have paracrine angiogenic activity. If the cell does not complete the lytic cycle (abortive lytic replication = Lytic*), it can become transformed due to the carcinogenicity of lytic gene expression, including *vGPCR*-mediated immortalization, and switch to the latent status with shutdown of lytic gene expression including *vGPCR*. This leads to strong VEGF dependence that should be supplied paracrinally.

activation. Such a private or internal autocrine loop has recently been described for endothelial lineage bone marrow hematopoietic progenitors (Gerber et al., 2002). The other possibility is that *vGPCR*, as described for other GPCRs, could directly transactivate KDR tyrosine phosphorylation (Pierce et al., 2001).

The finding that KSHV, an endothelial cell-transforming virus, encodes a gene with potential for endothelial immortalization and KDR autocrine activation has important implications for KS pathogenesis and the identification of new therapeutic approaches for AIDS-KS. It is consistent with new reports showing the molecular and pathogenic alterations that *vGPCR* causes specifically in endothelial lineage cells in vitro (Polson et al., 2002) and in vivo. Using avian retrovirus-mediated gene transfer to mice endothelium, it was recently found that *vGPCR* is the only KSHV gene able to provoke KS-like lesions and that it can paracrinally support KSHV latent gene tumorigenesis (Montaner et al., 2003).

Our finding that *vGPCR* expression increased VEGF secretion in immortalized human endothelial cells (Figure 6) supports the notion that in lytically infected cells of the KS lesion, *vGPCR* could activate paracrine angiogenesis and spindle cell proliferation (Bais et al., 1998; Cesarman et al., 2000). It also explains the ability of *vGPCR*-expressing endothelial cells for paracrine activation of angiogenesis and the promotion of the tumorigenic potential of latent genes (Montaner et al., 2003).

Assigning a role for a KSHV angiogenic gene such as *vGPCR*

in direct oncogenesis (Bais et al., 1998) or in the initiation of KS sarcomagenesis (Montaner et al., 2003; Hayward, 2003) is complicated by the fact that it is an early lytic gene not expressed in latently infected spindle cells composing the majority of the malignant cells of KS tumors (Ganem, 1998b; Kirshner et al., 1999; Staskus et al., 1997). However, at the same time, there are reports indicating that expression of the KSHV latent program found in malignant KS cells cannot transform endothelial cells (Lagunoff et al., 2002), as well as recent findings showing the inability of KSHV latent genes to initiate KS sarcomagenesis by endothelial infection in mice (Montaner et al., 2003). This paradox indicates that expression of KSHV oncogenic lytic genes may not always lead to viral progeny and cell killing and indicates that not all KSHV genes that participate in the oncogenesis process are expressed in the malignant KS cells. The mechanism that we describe for *vGPCR* immortalization involving KDR autocrine activation is compatible with such a modality of hit and run oncogenicity (Figure 7B), and it is supported by data showing that shutdown of *vGPCR* signaling in vGPCR-EC is compensated by external VEGF (Figure 7A). According to our findings, the lack of expression of *vGPCR* in transformed cells of KS lesions latently infected with KSHV could be compensated by paracrine VEGF, which could be provided in part by lytically infected cells expressing *vGPCR* (Bais et al., 1998; Montaner et al., 2003). The high levels of KDR expression and VEGF dependence of KS lesions (Brown et al., 1996; Cornali et al., 1996) and some KSHV-transformed endothelial cells (Flore et al., 1998; Masood et al., 2002) can be explained on the basis of this mechanism of *vGPCR* autocrine immortalization followed by *vGPCR* shutdown (Figure 7B). In addition to VEGF, KSHV latent expression could compensate anti-senescent and anti-apoptotic effects of *vGPCR*. *LANA* is known to promote telomerase transcription (Knight et al., 2001), while *vFLIP* can suppress apoptosis by NFkB activation (Liu et al., 2002). A similar mechanism of hit and run autocrine immortalization has been proposed for *HTLV-1 Tax*, which immortalizes T cells by creation of an IL-2/IL-2R autocrine loop, but is deleted from the provirus in HTLV-1-transformed IL-2-dependent adult T cell lymphoma cells (Cesarman and Mesri, 1999; Yoshida, 1999).

The model depicted in Figure 7 predicts that expression of KSHV lytic phase angiogenic genes and signaling oncogenes such as *K1* (Lagunoff et al., 1999; Lee et al., 1998), *vIL-6* (Aoki et al., 1999), and *vGPCR* in KSHV-infected cells that do not complete the lytic cycle is an event with high oncogenic risk. The probability of such an event is consistent with the low incidence of KS in the general infected population (Antman and Chang, 2000) and the low transforming potential of the virus (Flore et al., 1998; Lagunoff et al., 2002). The probability of abortive lytic infections such as the one described in Figure 7B is greatly increased with viral reactivation and de novo infection with KSHV. Accordingly, KS incidence increases with post-transplant immunosupression and AIDS (Gallo, 1998). These pathologies are associated with decreases in immune surveillance, inflammation, or the presence of HIV angiogenic molecules such as Tat, which have been related to increased KSHV reactivation (Antman and Chang, 2000; Ensoli et al., 2001). Conversely, drugs that block KSHV replication such as gancyclovir, and immune recovery, although they do not act in latently infected cells, have been shown to reduce KS incidence among AIDS patients (Antman and Chang, 2000).

Our results show that G protein-coupled receptor signaling can promote an anti-senescence response with lifespan extension in endothelial cells, which is associated with KDR expression and activation. This points to a novel mechanism for the regulation of angiogenesis by GPCRs in general and angiogenic chemokines in particular. The observation that *vGPCR* could determine the ability of KSHV for human endothelial immortalization and KDR activation warrants further studies on the role of this viral gene in KS pathogenesis and sets forth the possibility of targeting *vGPCR* and the VEGF-KDR activation axis to treat and prevent KSHV-induced oncogenesis.

Experimental procedures

Vector construction

The retroviral vectors used were a modification of the *pLXCN* vector (CMV IE driving expression) from Clontech in which the polylinker was expanded (*pLXCN2.0*). The two different inserts used in this paper (*HA-vGPCR* and *EGFP*) were subcloned into the HindIII/NotI sites of the pLCNX2.0 polylinker. *HA-vGPCR* coding HindIII/NotI fragment was taken from the *pCEFLHA-vGPCR* vector (Bais et al., 1998). *EGFP* coding HindIII/NotI fragments were taken from *p-N1-EGFP* vector (Clontech).

Retroviral transduction of HUVEC

Retroviral stocks were obtained using the Ampho-Phoenix cells from Dr. Garry Nolan, Stanford University, obtained through ATCC, following the protocols of public access at the Nolan Lab (website http://www.stanford.edu/group/nolan/). Passage 3 and 4 HUVEC obtained routinely at S.R. Lab using standard methods were infected with retroviral stocks in the presence of polybrene (4 ug/ml). Once expression of the *EGFP* transgene was verified, cells were passaged into a G418 containing medium.

Culture of HUVEC and transduced cells

HUVEC were cultured in gelatin-coated plates in ECM medium: M199 (Bio-Whittaker), 20% FCS (Gemini), 2 ng/ml of bFGF (PeProtech) and antibiotics (penicillin and streptomycin). Retrovirally transduced endothelial cells were grown in the same medium with the addition of G418 (Gibco-BRL). For serum free conditions, cells were incubated with X-VIVO 20 medium (Bio-Whittaker).

Immunohistochemistry

The cells were cultured on Labtek-Nunc chamber slides, coated with 0.2% gelatin, and live-stained with the antibodies as follows: After cooling to 4°C, the medium was removed and cells were washed once with DMEM before blocking for 30 min in DMEM, 10% goat serum at 4°C. After a wash with cold DMEM, the cell were incubated with the antibodies at 1 ug/ml (anti-HA: Covance-Babco, antiFlt-1 MoAb 6.12 and IMC-1C11 anti-KDR from Imclone) for 1 hr at 4°C and washed three times with cold DMEM. After washing, cells were fixed with 3.7% PBS formalin and permeabilized with methanol and acetone for 1 min each. After drying the slides, cells were rehydrated in PBS and blocked with PBS, 10% Goat serum. The secondary antibody, goat anti-mouse conjugated with alkaline phosphatase (Southern Biotech) was applied and incubated in 10% goat serum at 37°C for 1 hr. The slides were washed three times and the color reaction with Vector Red substrate kit was done according to the instructions provided by the manufacturer. The cells were rinsed with water and counterstained with 0.5 ug/ml DAPI in water for 5 min and rinsed with tap water. The slides were dehydrated and mounted. The pictures were taken on a Zeiss Axioskop or with a Zeiss confocal microscope.

Cytofluorometry

For detection of surface of KDR expression, cells dislodged by collagenase treatment were incubated with phycoerythrin (PE)-labeled high-affinity, non-neutralizing MoAbs to KDR clone 6.64 (ImClone Systems, New York, New York) (Peichev et al., 2000) for 20 min. The number of positive cells was compared to immunoglobulin G isotype control (PE; Immunotech, Marseille, France) and determined using Coulter Elite flow cytometer (COULTER, Hialeah, Florida).

Telomere length assay, telomerase activity, and TERT expression
Seven and a half micrograms of Genomic DNA of each cell line was digested 2 hr with 30 U of RsaI and 30 U of HinfII. Telomere length was measured by Southern blot using the telomere-specific oligonucleotide (TTAGG)4 and hybridized following the protocol described in Bryant et al. (1997). TRAP assay was performed using the TRAPeze Telomerase detection kit (Intergen Company, Cat. # S7700) and following a standard protocol provided with the kit. This assay measures the activity of telomerase in cell extracts by determining its ability to oligomerize telomeric oligonucleotides. The collection of oligomers is amplified by PCR using P-32-labeled primers, resolved in a 12.5% polyacrilamide gel and developed by autoradiography. *hTERT* expression was evaluated by RT-PCR using Superscript RT (GIBCO-BRL) and standard techniques. The TERT-specific primers employed were: 5′ primer CGGAAGAGTGTCTGGAGCAA, 3′ primer GGATGAAGCGGAGTCT GGA. For β-actin amplification, we employed primers supplied by Stratagene.

Thymidine incorporation
Cells were seeded in 96-well plates, coated with 2% gelatin, at 10,000 cells per well. After overnight growth, the cells were starved in X-VIVO 20 with 0.5% BSA and 3-H Thymidine (NEN) was added. After 24 hr incubation, the cells were frozen twice at −70°C to break up the cells. The cellular DNA was precipitated on glass fiber filters (Wallac) and the 3-H activity was measured in a scintillation counter.

Cell survival and apoptosis assays
To measure cell survival, confluent cultures in 24-well plates were incubated for the indicated times with the different additions either in SFM (X-VIVO 20) or in ECM (see specific figures). IP10, Gro-α, and VEGF were obtained from PeProtech, the PTK787 (Wood et al., 2000) was a gift from Novartis to S.R. Cells were counted after trypsinization using a coulter counter. For the experiments of Figure 6, the amount of viable cells was quantified by using the wst-1 cell proliferation reagent. In this case, instead of counting the cells, the assay media were replaced by ECM containing wst-1 (1:10 dilution), incubated for 30 min at 37°C in a cell incubator, and the optical density (O.D.) was measured at 450 nm. Apoptosis was determined using the Cell Death Detection ELISA-plus kit from Roche, which measures Histone-associated DNA associated to nucleosome degradation, following the instructions from the manufacturer. The fold increase in apoptosis was calculated by normalizing the value of the O.D.s to the O.D. value of the control experiment as indicated in the figures. In this condition, the increase in O.D. is in a linear relationship with the number of apoptotic cells. Experiments were repeated at least three times for consistency.

Determinations of AKT activation
Subconfluent cell cultures in 6-well plates were incubated overnight (O.N.) in X-VIVO 20 medium. Cells were then incubated with Gro-α (Peprotech) at a 25 nM final concentration for 5, 10, 20, and 40 min. Cells were washed with ice-cold PBS and lysed in 1.1× Laemmli cracking buffer. After vortexing for 10 min, lysates were heated 5′ at 95°C. Samples were analyzed by Western blot developed with the following antibodies: AKT phosho-plus and AKT (phosphoplus antibody kit, Cell signaling, Cat.#9270).

Determinations of tyrosine kinase phosphorylation of KDR
Sub-confluent cell cultures of vGPCR-EC or HUVEC serum starved O.N. in X-VIVO 20 medium were treated or not with VEGF (Peprotech) 100 ng/ml for 10 min. PTK787 was added at 0.1 ug/ml 1 hr prior to VEGF stimulation. After stimulation, cells were washed in ice-cold PBS, 1 mM Na_3VO_4, with addition of a protease inhibitor cocktail (SIGMA), and lysed in Triton/NP40 lysis buffer (0.5% triton X100, 0.5% NP-40, 10 mM Tris [pH 7.5], 2.5 mM KCl, 150 mM Na Cl, 30 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 0.1% Protease inhibitor cocktail SIGMA). Cell lysates were vortexed for 30 min at 4°C and centrifuged at 10000 × g at 4°C for 10 min. Supernatants were evaluated for protein concentration by the Bradford method (BioRad) and used as cell lysates. Lysates containing equal amounts of protein were incubated 30 min at 4°C with protein A/G PLUS-agarose (Santa Cruz) for pre-clearing and then incubated O.N. at +4°C with Anti-Flk-1/KDR polyclonal antibody (Santa Cruz). After that, they were incubated with protein A/G PLUS-agarose (Santa Cruz) for 2 hr at +4°C, the precipitates were washed four times with lysis buffer without detergents and then resuspended in SDS-PAGE sample buffer, boiled at 95°C for 5 min, separated by SDS-PAGE, and transferred to nitrocellulose membrane. Phosphotyrosine was detected with a monoclonal antibody (Upstate) and developed with anti-mouse IgG peroxidase (Cappel) and an ECL kit (Amersham).

VEGF determination by ELISA
Human VEGF was measured in post culture supernatants obtained after 48 hr of incubation in ECM. VEGF was quantified using an R&D Quantikine kit. Samples were analyzed in duplicate. Experiments were repeated two times.

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