

Transgenic Expression of the Chemokine Receptor Encoded by Human Herpesvirus 8 Induces an Angioproliferative Disease Resembling Kaposi's Sarcoma

By Tong-Yuan Yang,* Shu-Cheng Chen,* Michael W. Leach,‡
Denise Manfra,* Bernhard Homey,§ Maria Wiekowski,* Lee Sullivan,*
Chung-Her Jenh,* Satwant K. Narula,* Stephen W. Chensue,||
and Sergio A. Lira*

From the *Department of Immunology and the ‡Department of Drug Safety and Metabolism, Schering-Plough Research Institute, Kenilworth, New Jersey 07033; the §Department of Immunology, DNAX Research Institute, Palo Alto, California 94304; and the ||Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109

Abstract

Human herpesvirus 8 (HHV8, also known as Kaposi's sarcoma [KS]-associated herpesvirus) has been implicated as an etiologic agent for KS, an angiogenic tumor composed of endothelial, inflammatory, and spindle cells. Here, we report that transgenic mice expressing the HHV8-encoded chemokine receptor (viral G protein-coupled receptor) within hematopoietic cells develop angioproliferative lesions in multiple organs that morphologically resemble KS lesions. These lesions are characterized by a spectrum of changes ranging from erythematous maculae to vascular tumors, by the presence of spindle and inflammatory cells, and by expression of vGPCR, CD34, and vascular endothelial growth factor. We conclude that vGPCR contributes to the development of the angioproliferative lesions observed in these mice and suggest that this chemokine receptor may play a role in the pathogenesis of KS in humans.

Key words: ORF74 • viral pathogenesis • animal model • angiogenesis • VEGF

Introduction

Kaposi's sarcoma (KS),¹ which was first described by Moriz Kaposi in 1872, may be clinically divided into four distinct forms: rare classical KS, more common posttransplant and AIDS-associated KS, and endemic KS, which is common in equatorial Africa (1). Histopathologically, this multifocal tumor is characterized by an angiogenic proliferation of mesenchymal cells with the formation of slit-like vascular spaces surrounded by spindle cells and infiltrating inflammatory cells. An infectious etiology for KS was first proposed in 1994, when Chang et al. (2) discovered a novel human herpesvirus (human herpesvirus 8 [HHV8], or KS-associated herpesvirus [KSHV]) during systematic DNA screening of KS lesions. In recent years, epidemiological and experimen-

tal studies have strengthened the link between this lymphotropic herpesvirus and KS by showing that HHV8 infection precedes development of tumors, tracks tightly with KS risk, and targets spindle cells (3–5). Furthermore, HHV8 encodes several genes with angiogenic and transforming properties that may be important for the development of KS (6). One such gene (ORF74) encodes a constitutively active viral G protein-coupled receptor (vGPCR) that binds several host CC and CXC chemokines (7, 8). Transient transfection of this receptor into fibroblasts causes intracellular accumulation of inositol phosphate (7), increased cellular proliferation, and expression of vascular endothelial growth factor (VEGF; reference 9), a potent angiogenic factor. Taken together, these results strongly suggest a link between HHV8 and KS in vivo, but they do not prove that HHV8 is sufficient for KS development nor define which HHV8 genes are relevant for pathogenesis. To start addressing the biological function of HHV8-encoded genes in vivo, we generated transgenic mice expressing vGPCR under the control of the human CD2 promoter (10).

Address correspondence to Sergio A. Lira, Dept. of Immunology, Schering-Plough Research Institute, 2015 Galloping Hill Rd., Kenilworth, NJ 07033. Phone: 908-740-3088; Fax: 908-740-3084; E-mail: sergio.lira@spcorp.com

¹Abbreviations used in this paper: HHV8, human herpesvirus 8; KS, Kaposi's sarcoma; RT, reverse transcriptase; VEGF, vascular endothelial growth factor; vGPCR, viral G protein-coupled receptor.

Materials and Methods

Generation of Transgenic Mice. The coding sequence for vGPCR was amplified from the genomic DNA of BC-1 cells (American Type Culture Collection) with primers TY42 (5'-GGAATTC-ACCACCATGGCGGCCGAGGATTTCC-3') and TY10 (5'-ATCCTGCAGGGGCTACGTGGTGGCGCCGACAT-3'). Sequencing of the amplified fragment showed it to be identical to the gene described by Cesarman et al. (3). The 1.0-kb vGPCR fragment was cloned into EcoRI-SmaI sites of a plasmid containing the human CD2 enhancer/promoter and locus control region (10). Generation and genotypic analyses of the animals were done as previously described (11). The resulting transgenic animals were kept under pathogen-free conditions.

RNA Analysis. RNA was extracted from tissues or sorted cells using Ultraspec RNA, following specifications from the manufacturer (Biotecx). Total RNA (20 µg) was denatured and blotted onto GeneScreen membrane (New England Nuclear). A 1.0-kb fragment of the vGPCR DNA was radiolabeled and was used as a probe in these experiments. For reverse transcriptase (RT)-PCR, cDNA was synthesized from 2 µg of total RNA primed with oligo-(dT)12-18 using the Superscript II Pre-amplification System (GIBCO BRL). 2-5 µl of cDNA was amplified in a 50-µl reaction volume under the following conditions: 94°C for 1 min and 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min and then incubated in a GeneAmp® PCR System (Perkin-Elmer Cetus). The oligonucleotide primers corresponding to the sense and antisense strands, respectively, were as follows: vGPCR, 5'-ATGGCGGCCGAGGATTTCCCTAACC-3' and 5'-AGGTACCTCACTAGACTGACGCAC-3'; and G3PDH, 5'-TGAAGGTGGTGTGAACGGATTTGGC-3' and 5'-CATGTAAGGCCATGAGGTCCACCAC-3'. The final PCR products were analyzed on a 2% agarose gel containing ethidium bromide.

Human IFN-γ-inducible Protein 10 Binding Assay. Thymocytes (10⁶ cells per point) were resuspended in binding buffer (50 mM Hepes, 1 mM CaCl₂, 5 mM MgCl₂, 0.5% BSA) and incubated with 0.1 nM of radiolabeled human IFN-γ-inducible protein (hIP-10; NEN Life Science Products) in the absence or presence of 100 nM of unlabeled human IP-10 in 96-well plates. After 2 h at room temperature, plates were spun down, and cell pellets were resuspended in binding buffer supplemented with 0.5 M NaCl. Cells were spun through 10% glycerol in binding buffer with 0.5 M NaCl. Cell pellets were frozen in liquid nitrogen, clipped, and counted. All experiments were performed in triplicate.

Histochemistry, Immunohistochemistry, Electron Microscopy, and In Situ Hybridization. Tissues were either fresh-frozen for cryosection or were fixed, processed, and stained with hematoxylin and eosin. For immunostaining, fresh-frozen sections were fixed with acetone, and rehydrated paraffin sections were subjected to antigen retrieval first. For transmission electron microscopy, tissues were collected in 2.5% glutaraldehyde and then submitted for standard osmium fixation and plastic embedding. Thin sections were placed on grids and examined using an LEO electron microscope (Zeiss Instruments). For immunohistochemical staining, anti-CD34 antibody was provided by Dr. A. Mantovani (Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy). Anti-CD3, anti-F4/80 (Serotec Ltd.), anti-B220, and anti-CD11b/Mac-1 (PharMingen) were also used in the experiments. Binding of the antibody was detected with a Vectastain Elite ABC kit (Vector Labs., Inc.). DAB and AEC kits from Vector were used as substrates for peroxidase. Hematoxylin was used for counterstaining. In situ hybridization was carried out as described (12). Sense and antisense ³³P-labeled riboprobes were transcribed

from cDNA templates of vGPCR (from nucleotides 473-1,075) or VEGF (from nucleotides 91-429) with either T7 or T3 polymerase (Boehringer Mannheim).

Bone Marrow Transplantation. Sterile bone marrow cells were flushed out from femurs of CDVG (CD2 promoter-vGPCR) and control mice. Single-cell suspensions (10⁷ cells) were injected into the tail veins of lethally irradiated (1,100 rads for 5 min) B6D2 F1 mice (6-8 wk old).

Cell Sorting. Single-cell suspensions were prepared from CDVG spleens by passage through a 100-µm nylon cell strainer (Falcon-Becton Dickinson) in RPMI medium containing 5% FCS. RBCs were lysed, and the remaining cells were washed and resuspended in 1× PBS containing 0.5% BSA and 2 mM EDTA (buffer A). To prevent nonspecific binding of the antibodies, Fc (CD16/CD32) receptors were blocked by incubating in buffer A containing 5 µl of Fc Block (PharMingen) and 300 µg/ml of whole mouse IgG per 10⁶ cells for 10 min at room temperature. Dead and apoptotic cells were removed using the Dead Cell Removal kit from Miltenyi Biotec as per the manufacturer's recommendations. Directly conjugated microbeads (Miltenyi Corp.) were used to isolate cell populations expressing Mac-1 (CD11b), B220, and Thy1.2. To isolate NK cells, we used DX5 antibodies conjugated with biotin and subsequently used streptavidin microbeads as per the manufacturer's recommendations. Flow cytometric analysis of these sorted cells showed that they were 83-92% pure. 10⁷ cells in each case were used for RNA isolation.

CD2⁺ cells from tumors were isolated as follows: Solid tumors were excised from CDVG mice, minced, and incubated for 20 min at 37°C in RPMI containing 5% FCS, 10 mM Hepes/5% FBS, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.5 mg/ml collagenase D (Boehringer Mannheim), and 35 U/ml DNase I (Sigma Chemical Co.). This suspension was passed through a wire mesh to eliminate debris, and the incubation was continued for an additional 20 min at 37°C. At the end of the incubation, cells were passed through a 100-µm mesh and pelleted at 1,000 rpm for 10 min at 4°C. The RBCs were lysed, the Fc receptors were blocked, and the dead cells were removed as described above. The cells were pelleted and resuspended in buffer A containing biotin-conjugated anti-CD2 at a predetermined concentration, and the suspension was incubated for 15 min at 4°C on a rotator. The cells were washed in buffer A, and the CD2⁺ cells were isolated using Strep microbeads (Miltenyi Corp.) as per the manufacturer's recommendations. Sorted cells were 85-90% pure.

Results

Expression of vGPCR within Hematopoietic Cells of Transgenic Mice. Because HHV8 is a member of the lymphotropic subgroup of herpesviruses (6) and its presence has been detected in blood mononuclear cells (13), we reasoned that cells of hematopoietic origin may represent a good experimental system to study vGPCR effects in vivo. To this end, we used an expression vector containing the human CD2 promoter, which primarily targets gene expression to hematopoietic cells (Fig. 1 A). We obtained seven transgenic founders, from which five transgenic lines were derived. We refer to these transgenic mice as CDVG (CD2 promoter-vGPCR) mice. Transgene expression was initially evaluated by Northern blot analysis. The vGPCR transcripts were detected in thymi and bone marrow of

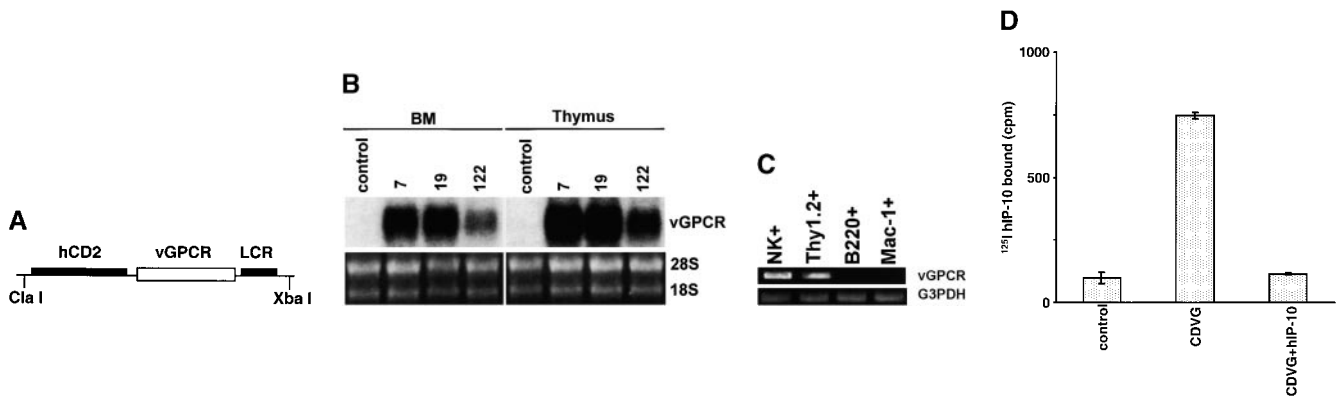


Figure 1. Generation and expression analysis of CDVG-transgenic mice. (A) Structure of the CDVG transgene. (B) Northern blot analysis of bone marrow (BM) and thymus RNA from control and CDVG-transgenic mice (transgenic lines 7, 19, and 122). The BM blot was exposed three times longer than the thymus blot. Bottom panel shows the ethidium bromide-stained RNA gels. (C) RT-PCR analysis of transgene expression in subsets of cells isolated from the spleens of transgenic mice. Expression of the transgene was detected in NK (NK+) and T (Thy1.2+) but not in B (B220+) and myeloid (Mac-1+) cell-enriched populations. (D) Radiolabeled hIP-10 binding to the transgenic and control thymocytes. Binding of radiolabeled hIP-10 to transgenic thymocytes can be displaced by cold hIP-10 (CDVG+hIP-10).

transgenic but not of control mice, consistent with the well documented pattern of transgene expression driven by the CD2 promoter (reference 14; Fig. 1 B). To investigate the transgene expression in different hematopoietic cells, we isolated leukocyte subsets by magnetic sorting, extracted RNA, and performed RT-PCR. As shown in Fig. 1 C, vGPCR transcripts could be amplified from NK and T cell-enriched populations but not from B cells or Mac-1+ cells. These results point to a predominant expression of the transgene in T and NK cells. Next, we examined the expression of vGPCR at the cell surfaces of transgenic thymocytes by measuring binding of radiolabeled human (h)IP-10, a chemokine known to inhibit constitutive vGPCR signaling (15). Thymocytes from vGPCR-transgenic mice showed more than a fivefold increase of radiolabeled hIP-10 binding compared with control thymocytes (Fig. 1 C). Binding of hIP-10 to transgenic thymocytes could be reduced by competition with unlabeled hIP-10. Increased binding of hIP-10 to transgenic thymocytes was not related to binding to its murine homologue receptor, CXCR3, because mCXCR3 expression was not detected in transgenic thymi by Northern blot analysis (data not shown). These results confirmed the expression of vGPCR within hematopoietic cells of transgenic mice.

CDVG Mice Develop Angioproliferative Disease within Multiple Organs. Mice expressing vGPCR were normal at birth. However, within the first 30–90 d of life, all transgenic mice from three independent lines (9, 19, and 122) developed lesions that initially consisted of erythematous to purplish maculae and plaques that subsequently progressed to purple nodules and eventually to overt tumors on the ears, tail, nose, and paws (Fig. 2, A–E). Erythematous nodules were also seen in skeletal muscle and in the walls of the small and large intestine (Fig. 2 F). Microscopically, the CDVG tumors were multicentric angioproliferative lesions that were nodular and grew between or invested normal structures of skin (Fig. 3 A). Similar lesions were detected occasionally in heart, skeletal muscle, and the submucosa

and muscularis propria of the small and large intestine. They were characterized by sarcomatoid appearing sheets and fascicles of spindled cells forming slit-like and round vascular spaces that contained erythrocytes (Fig. 3, B and C). Mitotic figures were uncommon, and necrotic cells were rare. In addition, the CDVG lesions showed strong expression of CD34, an endothelial cell marker present in angiogenic tumors, including KS tumors (Fig. 3 D; reference 16).

To confirm the light microscopic impression and to exclude the possibility of other vascular-rich neoplasms, lesions from multiple sites were examined by transmission electron microscopy. At the ultrastructural level, the lesions from different sites were similar and composed of endothelial cells forming vascular spaces within a loose collagen matrix. Erythrocytes were often present within vascular lumina (Fig. 4, top panels). The endothelial cells lining vascular spaces showed typical features of tight junctions and micropinocytotic vesicles. Pericytes were present in variable numbers. As described for human KS, Weibel-Palade bodies were absent (17). More primitive spindled cells were identified in the interstitial spaces. These less differentiated cells had a cytoplasm with prominent rough endoplasmic reticulum with scattered mitochondria and lysosomes. These were observed in various stages of lumen formation and thus appeared to be endothelial cell precursors. There was no evidence of epithelial, mesothelial, fibrohistiocytic, rhabdoid, or neuroid differentiation.

Scattered throughout the vascular lesions were hemosiderin deposits and variable numbers of mixed inflammatory cells, including macrophages, eosinophils, mast cells, neutrophils, and lymphocytes. Occasional extravasated erythrocytes and inflammatory cells were also present in the interstitium (Fig. 4, top panels). To further phenotype the leukocytes present within the lesions, we performed immunohistochemistry (Fig. 4, bottom panels). The lymphocytes detected by light and electron microscopy were mostly T cells. Other lymphocytes, such as B cells, were also found, but at lower frequency. Macrophages, visualized by F4/80

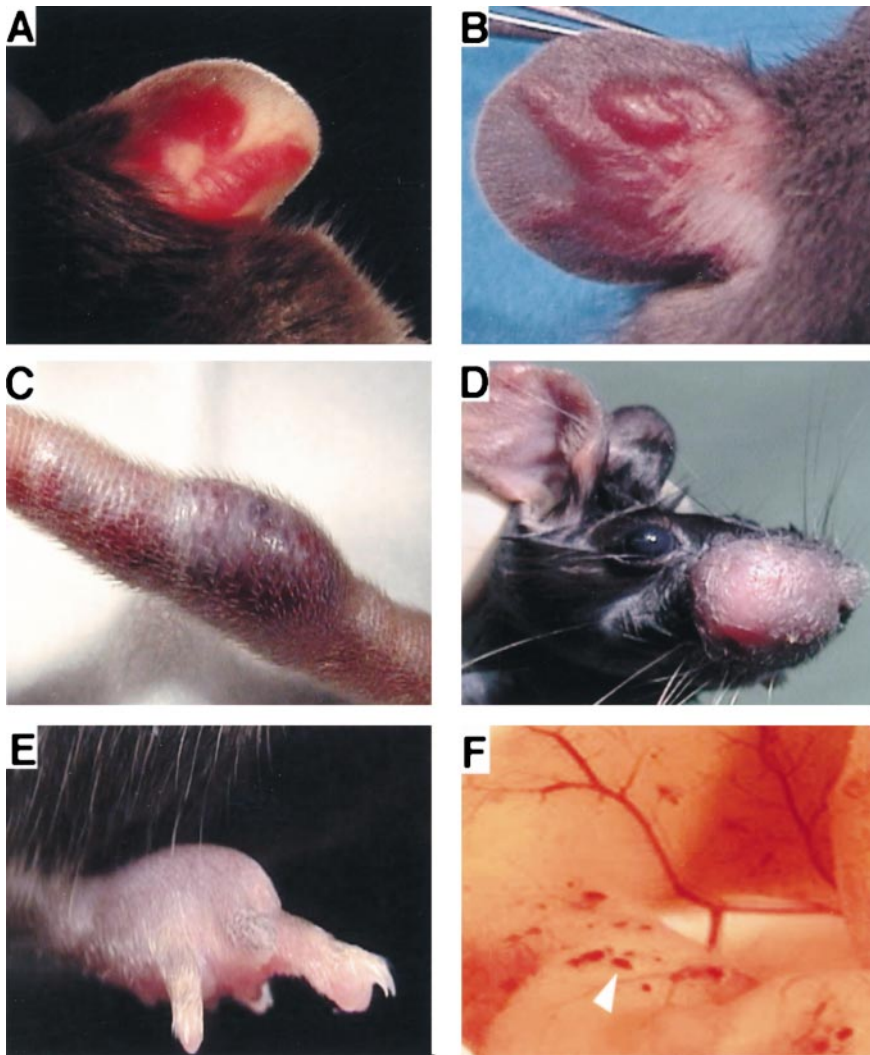


Figure 2. KS-like lesions in CDVG-transgenic mice. Shown are (A) an erythematous, sharply-bordered plaque on the ear; (B) later lesions showing raised purplish or erythematous nodules and tumors on the ear, (C) tail, (D) nose, and (E) paw; and (F) erythematous nodules in wall of the small intestine (one is indicated by the white arrowhead).

immunostaining, could also be found within the lesions. These results confirm the presence of several types of inflammatory cells within the lesions. Taken together, the histologic and ultrastructural features described above suggested a Kaposi-like angioproliferative neoplasm with infiltration of inflammatory cells.

vGPCR and VEGF Are Expressed within Angioproliferative Lesions. To investigate whether the vascular changes observed in the CDVG mice were associated with the expression of vGPCR, we performed RT-PCR and in situ hybridization studies. Transgene transcripts were detected in samples with macroscopic lesions (ear, muscle) but were not found in transgenic tissue samples that lacked lesions (Fig. 5 A and Fig. 5 C, left panel). As the transgene expression was directed by CD2 regulatory elements, we investigated whether CD2⁺ cells could be found within the lesions. To this end, we isolated CD2⁺ cells from the tumors using magnetic sorting. Next, we investigated if these CD2⁺ cells expressed the transgene by RT-PCR analysis. As shown in Fig. 5 B, transgene expression could be detected by RT-PCR in these CD2⁺ cells. To investigate the distribution of the vGPCR-expressing cells, we performed in situ hybridiza-

tion. Interestingly, similar to human KS (18), vGPCR expression can be detected in a subset of cells within the lesion (Fig. 5 C, left panel). Morphologically, most of these cells were either round or oval-shaped; we only rarely detected expression in spindle-shaped cells.

The fact that vGPCR expression took place in some but not all cells within the tumor could suggest that vGPCR expression may influence tumor growth by an indirect mechanism. In vitro, vGPCR induces the expression of VEGF (9), a potent angiogenic factor. Expression of VEGF by a small number of cells could conceivably result in significant vascular proliferation. To determine whether VEGF was expressed within the transgenic lesions, we performed in situ hybridization. We found that, within the same lesion, VEGF was expressed by a few scattered cells, in a pattern of expression that closely resembled that of the transgene (Fig. 5 C, right panel). These results suggest that cells expressing vGPCR may also express VEGF and that VEGF may be involved in the development of the vascular lesions. It is unlikely, however, that VEGF alone accounts for all of the phenotypes observed in CDVG mice, because transgenic animals expressing VEGF develop milder angiogenic changes (19, 20) and, more im-

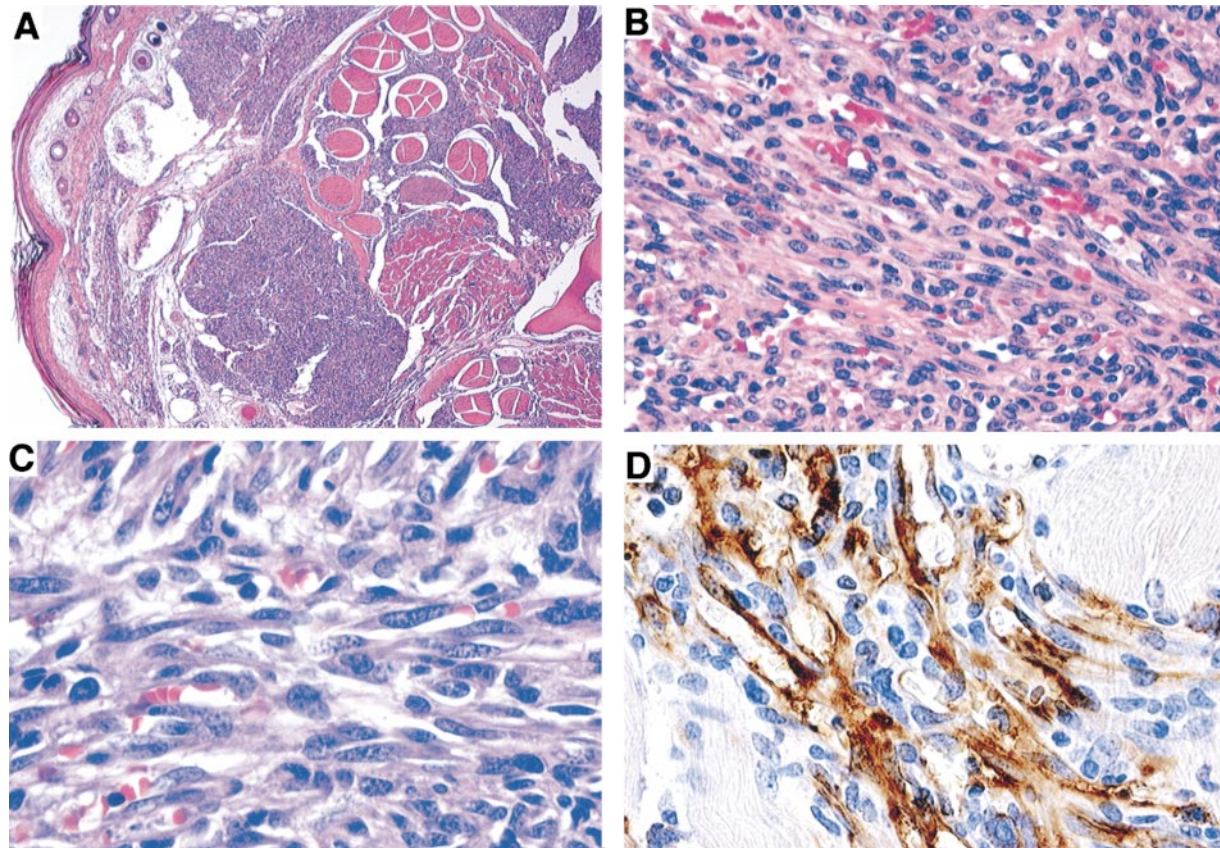


Figure 3. Microscopic changes in CDVG mice. (A) Tail. Multiple nodular angioproliferative tumors located in the dermal compartment of the skin. (B) Tail. Large numbers of spindle-shaped cells arranged in interlacing fascicles. Blood vessels are lined by plump endothelial cells and surrounded by oval to spindle-shaped cells. (C) Ear. Border between interlacing fascicles of spindle-shaped cells. There are irregular vascular clefts between cells that contain erythrocytes. (D) Immunohistochemical staining of a typical angioproliferative lesion with anti-CD34. Numerous positively stained cells are present.

portantly, the lesions described in VEGF-transgenic mice did not evolve into the sarcoma-like lesions observed here. Additional factors may thus be involved in the generation of the lesions observed in CDVG mice.

Animals Transplanted with Bone Marrow from CDVG Mice Develop Angioproliferative Disease. In a separate set of experiments, we were able to observe KS-like disease in animals in which vGPCR expression was driven by a CMV enhancer- β -actin promoter element (our unpublished observations). Three founders expressing vGPCR developed a disease that was clinically and histologically indistinguishable from the one described above. However, we were unable to propagate lines from these founders, due to either extreme mosaicism or early lethality of the positive offspring. The occurrence of disease in these animals could be due to expression of vGPCR in multiple tissues, raising the possibility that the disease observed in the CDVG mice could be due to ectopic expression of the transgene. To further demonstrate that the disease-causing cells in the CDVG mice had a hematopoietic origin and to rule out the possibility that the phenotype observed in the CDVG mice was due to ectopic expression of the transgene, we transplanted bone marrow from CDVG or control mice into lethally irradiated wild-type

animals. In contrast to mice receiving control bone marrow, CDVG bone marrow-transplanted mice clearly showed the presence of the transgene in bone marrow cells (Fig. 6 A). 7 mo after transplantation, the recipients of control bone marrow ($n = 3$) were disease free, but two out of three of the animals transplanted with CDVG bone marrow developed small purplish nodules at the bases of their tails (Fig. 6 B). Clinically and histopathologically, these lesions shared all of the characteristics of tumors seen in CDVG mice. Angioproliferative lesions were nodular and grew below a normal epidermis within the dermal compartment of the skin (Fig. 6 C). As in the donor strain, the tumors showed spindle-shaped cells forming slit-like vascular spaces that contained erythrocytes and variable numbers of mixed inflammatory cells (Fig. 6 D). These results, therefore, demonstrate that this angioproliferative disease can be transferred by bone marrow transplantation, suggesting that cells of hematopoietic origin play a crucial role in its pathogenesis.

Discussion

Although epidemiological evidence suggests an infectious etiology for KS and clearly links this multicentric angiopro-

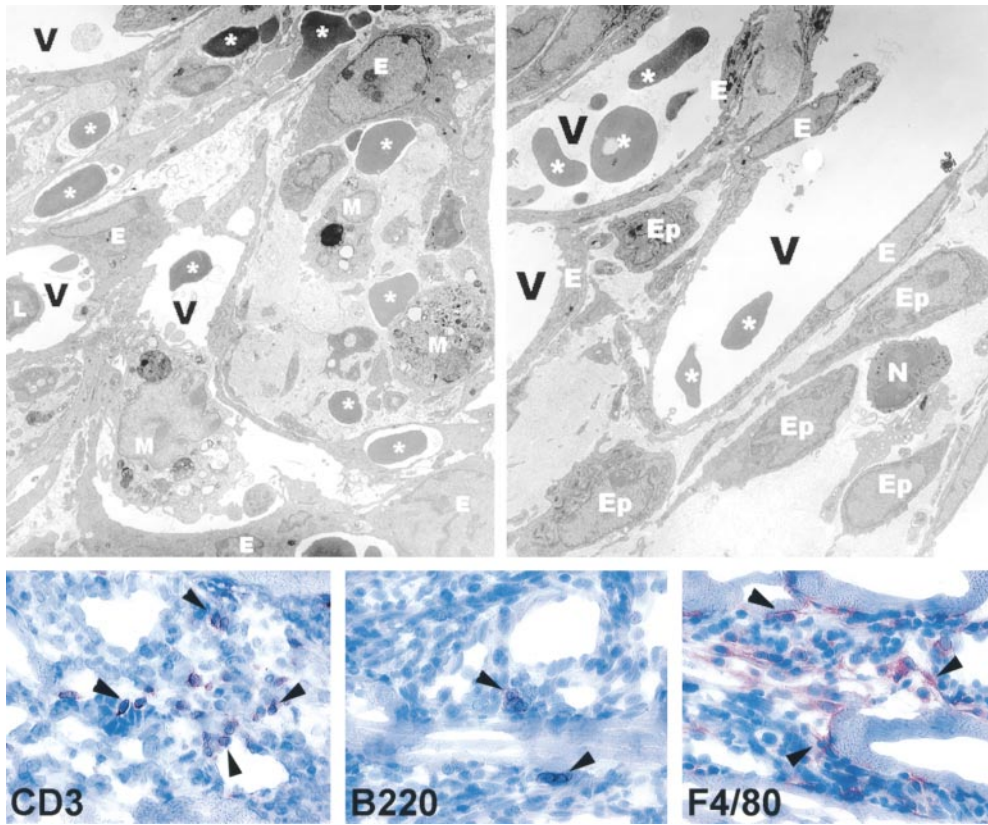


Figure 4. Analysis of angioproliferative lesions. Top panels: ultrastructural analysis of angioproliferative tumors. Panels depict representative areas taken from skin and pericardial tumors. Note numerous vascular spaces (V) lined by endothelial cells (E) and containing erythrocytes (*) and occasional lymphocytes (L). Extravasated erythrocytes are also present in interstitium with nearby macrophages (M), top left panel. Spindled endothelial cell precursors (Ep) are among vessels with occasional inflammatory neutrophils (N), top right panel. Bottom panels: immunohistochemical staining of angioproliferative lesions. Positive cells are stained red. Notice the presence of cells staining for CD3, F4/80, and B220 (arrowheads).

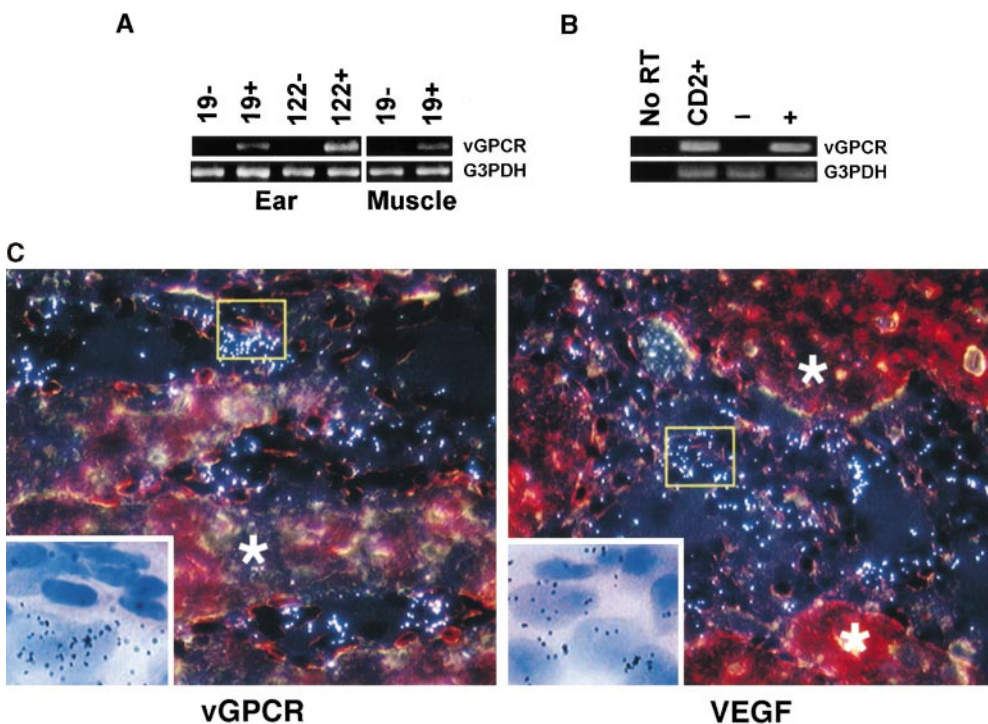


Figure 5. Detection of the expression of vGPCR and VEGF within angioproliferative lesions of CDVG mice. (A) RT-PCR analysis of vGPCR expression within ears and muscle from animals in two separate transgenic lines of CDVG mice, 19 and 122. Samples obtained from tissues with gross lesions (+) and without lesions (-) were used. Amplification of a segment of G3PDH was used as an internal control for the reactions. (B) Magnetically sorted CD2⁺ cells found within tumors express vGPCR. Positive (+) and negative (-) controls for this reaction were thymi from transgenic and control mice, respectively. No signal was observed in CD2 samples not reverse transcribed, indicating that the signal was derived from transgenic transcripts rather than genomic DNA. (C) In situ hybridization analysis of the expression of vGPCR and VEGF within the muscle of CDVG mice, dark field. The silver grains are only associated with infiltrating cells in the lesion. No signals found in the muscle cells (indicated by white *). Inset, higher magnification of the boxed area, bright field.

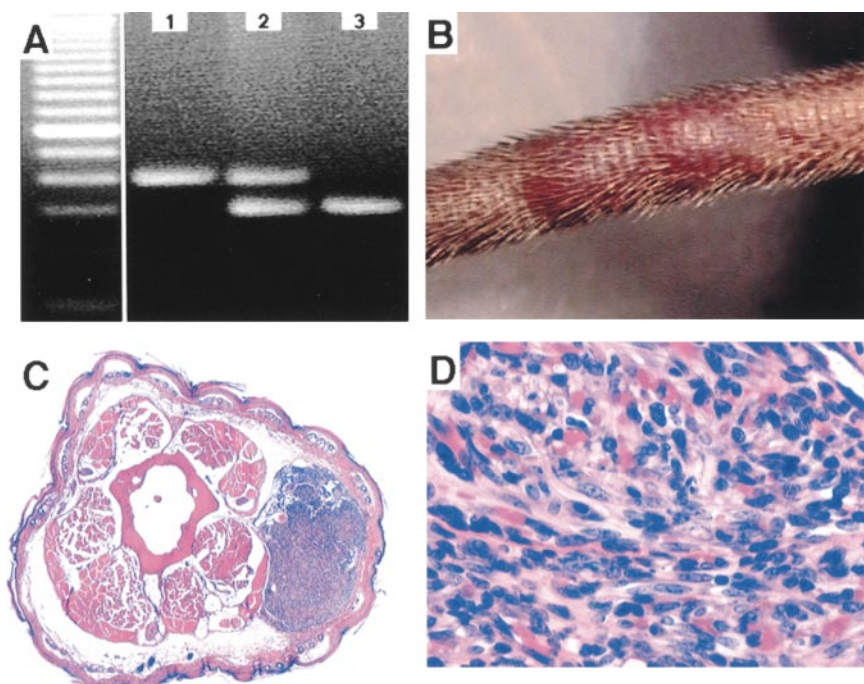


Figure 6. Transplantation of bone marrow cells from CDVG mice induces angioproliferative tumors in recipient mice. (A) PCR analysis of the vGPCR gene in genomic DNA of bone marrow of wild-type (lane 1) and CDVG bone marrow-transplanted mice (lane 2). Lane 3 represents the vGPCR control plasmid (250 bp, lower band). The upper band is the 383-bp internal control band from the *LDL* gene. (B) Tail. Purplish nodular tumor developed 7 mo after bone marrow transplantation. (C) Tail. Nodular angioproliferative tumor within the dermis. (D) Tail. Formation of vascular clefts containing erythrocytes surrounded by spindle and inflammatory cells.

liferative disease with HHV8 infection, the functional role of this lymphotropic herpesvirus in the pathogenesis of KS has remained unclear (6, 21, 22). A major limitation in addressing this critical question has been the lack of an experimental animal model. To circumvent this limitation, we began a systematic analysis of the biological properties of HHV8-encoded genes using transgenesis. Here, we report that a single gene of HHV8 is sufficient to induce in mice an angioproliferative disease that bears striking similarity to human KS. Clinically, the lesions start as erythematous maculae or plaques and progress to violaceous, purplish nodules and tumors that develop predominantly in the skin and to a lesser extent in intestine, heart, and skeletal muscle. Histologically and ultrastructurally, these lesions show characteristics similar to those of human KS and Kaposiform hemangioendotheliomas. Lesions within the skin developed and spread within the dermis while sparing the overlying epidermis. Like KS, the nodular angioproliferative tumors formed slit-like vascular spaces containing erythrocytes and were surrounded by spindle cells and infiltrating inflammatory cells (16, 21, 23, 24). Finally, vGPCR, VEGF, and CD34, markers that have been detected in KS, are also detected within these lesions (18, 25–27).

The angioproliferative disease observed in the CDVG animals is most likely caused by cells of hematopoietic origin, because control mice transplanted with CDVG bone marrow cells develop disease. It is unclear at this point, however, whether the cell responsible for disease development is a common hematopoietic cell or a rare, immature cell, such as an endothelial cell precursor (28, 29). Independent of the precise nature of this cell, it is likely that it expresses CD2, as CD2 regulatory elements were used to target expression of the transgene. In mice, CD2 is expressed in thymocytes, T cells, NK cells, and B cells. The CD2

promoter used in our experiment, however, primarily targets a subset of these cells, more specifically T lymphocytes (10) and NK cells. Thus, if the disease is caused by a common hematopoietic cell, the most likely candidates for inducing it would be T lymphocytes (mature or immature) and NK cells. A number of other hematopoietically derived cells are also present in the lesions, including B cells and macrophages. However, Northern blot analysis of B cell-rich tissues such as Payer's patches (data not shown) and RT-PCR analysis of B cells extracted from the spleen failed to show transgene expression, indicating that these cells do not express the transgene at appreciable levels. Other cells found in the lesions include macrophages, but preparations of peritoneal macrophages (not shown) and Mac-1⁺ cells from spleens were negative for transgene expression. We cannot exclude, however, that low level expression of vGPCR by these or other cell populations could be important for the pathogenesis of the KS-like disease observed here. The resolution of this matter will clearly require the development of specific reagents to immunophenotype the cell expressing the transgene within the lesion and the demonstration through genetic and adoptive transfer experiments that expression of vGPCR by this cell can lead to development of disease.

Based on the results obtained with the bone marrow transplants, we propose that disease development involves homing of circulating vGPCR-expressing cells to different tissues. These cells may have physiologically homed to these tissues or may have abnormally migrated due to the constitutive expression of this viral chemokine receptor on their surfaces. Once these vGPCR-expressing cells homed to their appropriate environment, they may have contributed to the intense vascular proliferation and inflammatory cell recruitment that we observed. Although we cannot

rule out that the incoming cell may proliferate as function of vGPCR expression, we favor an indirect, paracrine mechanism for the vascular proliferation, because few of the cells within the lesion are expressing vGPCR. One of these paracrine factors is VEGF, which has been shown to be an important requirement for the growth of cells latently infected with HHV8 and development of KS in humans (9, 27, 30).

It has been proposed that in KS (contrary to other models of virus-induced tumorigenesis), the growth of latently infected cells may be critically dependent on paracrine factors produced by neighboring cells undergoing a switch from latency to the lytic cycle (18, 31). Studies done in cell culture and in KS tumors have shown that vGPCR, vIL-6, viral macrophage inflammatory protein I, and vBcl2 are expressed by few infected cells in the early lytic phase of the life cycle of HHV8 (18, 31), but it has been unclear whether these lytic cycle products could account for any of the pathogenesis of KS. Results reported here show that vGPCR is sufficient to induce in mice an angioproliferative disease that has remarkable similarities to KS, adding additional support to the notion that HHV8 is an etiologic agent of this disease. Furthermore, they suggest that paracrine mechanisms triggered by the expression of this chemokine receptor play an important role in the development of angioproliferative diseases such as KS. Taken together, these observations implicate vGPCR expression during the lytic phase of HHV8 infection as an important factor in the pathogenesis of KS and suggest that vGPCR may represent a novel target for therapeutic intervention. Further studies in these directions should be facilitated by the development of the animal model described in this report.

We are grateful to Dr. C. Strader for critical reading of and suggestions for the manuscript. We thank Drs. D. Kioussis and A. Mantovani for reagents and Petronio Zalamea, Linda Hamilton, Yuetian Chen, Lisa Tardelli, Channa Young, and Mary Ann Cox for expert technical assistance. We also thank Susan Stamper and Lynn St. Dennis for processing tissues for electron microscopy.

Submitted: 5 August 1999

Revised: 9 November 1999

Accepted: 12 November 1999

References

- Slavin, G., H.M. Cameron, and H. Singh. 1969. Kaposi's sarcoma in mainland Tanzania: a report of 117 cases. *Br. J. Cancer.* 23:349-357.
- Chang, Y., E. Cesarman, M.S. Pessin, F. Lee, J. Culpepper, D.M. Knowles, and P.S. Moore. 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science.* 266:1865-1869.
- Cesarman, E., R.G. Nador, F. Bai, R.A. Bohenzky, J.J. Russo, P.S. Moore, Y. Chang, and D.M. Knowles. 1996. Kaposi's sarcoma-associated herpesvirus contains G protein-coupled receptor and cyclin D homologs which are expressed in Kaposi's sarcoma and malignant lymphoma. *J. Virol.* 70: 8218-8223.
- Arvanitakis, L., E.A. Mesri, R.G. Nador, J.W. Said, A.S. Asch, D.M. Knowles, and E. Cesarman. 1996. Establishment and characterization of a primary effusion (body cavity-based) lymphoma cell line (BC-3) harboring Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) in the absence of Epstein-Barr virus. *Blood.* 88:2648-2654.
- Nador, R.G., E. Cesarman, A. Chadburn, D.B. Dawson, M.Q. Ansari, J. Said, and D.M. Knowles. 1996. Primary effusion lymphoma: a distinct clinicopathologic entity associated with the Kaposi's sarcoma-associated herpes virus. *Blood.* 88:645-656.
- Ganem, D. 1997. KSHV and Kaposi's sarcoma: the end of the beginning? *Cell.* 91:157-160.
- Arvanitakis, L., E. Geras-Raaka, A. Varma, M.C. Gershengorn, and E. Cesarman. 1997. Human herpesvirus KSHV encodes a constitutively active G-protein-coupled receptor linked to cell proliferation. *Nature.* 385:347-350.
- Geras-Raaka, E., L. Arvanitakis, C. Bais, E. Cesarman, E.A. Mesri, and M.C. Gershengorn. 1998. Inhibition of constitutive signaling of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor by protein kinases in mammalian cells in culture. *J. Exp. Med.* 187:801-806.
- Bais, C., B. Santomasso, O. Coso, L. Arvanitakis, E.G. Raaka, J.S. Gutkind, A.S. Asch, E. Cesarman, M.C. Gershengorn, and E.A. Mesri. 1998. G-protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus is a viral oncogene and angiogenesis activator. *Nature.* 391:86-89.
- Zhumabekov, T., P. Corbella, M. Tolaini, and D. Kioussis. 1995. Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. *J. Immunol. Methods.* 185:133-140.
- Lira, S.A., R.A. Kinloch, S. Mortillo, and P.M. Wassarman. 1990. An upstream region of the mouse ZP3 gene directs expression of firefly luciferase specifically to growing oocytes in transgenic mice. *Proc. Natl. Acad. Sci. USA.* 87:7215-7219.
- Lugo, D., J. Roberts, and J. Pintar. 1989. Analysis of proopiomelanocortin gene expression during prenatal development of the rat pituitary gland. *Mol. Endocrinol.* 3:1313-1324.
- Whitby, D., M.R. Howard, M. Tenant-Flowers, N.S. Brink, A. Copas, C. Boshoff, T. Hatzioannou, F.E. Suggett, D.M. Aldam, A.S. Denton, et al. 1995. Detection of Kaposi's sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. *Lancet.* 346: 799-802.
- Lang, G., D. Wotton, M.J. Owen, W.A. Sewell, M.H. Brown, D.Y. Mason, M.J. Crumpton, and D. Kioussis. 1988. The structure of the human CD2 gene and its expression in transgenic mice. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:1675-1682.
- Geras-Raaka, E., A. Varma, H. Ho, I. Clark-Lewis, and M.C. Gershengorn. 1998. Human interferon γ -inducible protein 10 (IP-10) inhibits constitutive signaling of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor. *J. Exp. Med.* 188:405-408.
- Jones, R.R., G. Orchard, B. Zelger, and E.W. Jones. 1995. Immunostaining for CD31 and CD34 in Kaposi sarcoma. *J. Clin. Pathol.* 48:1011-1016.
- Kostianovsky, M., Y. Lamy, and M.A. Greco. 1992. Immunohistochemical and electron microscopic profiles of cutaneous Kaposi's sarcoma and bacillary angiomatosis. *Ultrastruct. Pathol.* 16:629-640.
- Kirshner, J.R., K. Staskus, A. Haase, M. Lagunoff, and D. Ganem. 1999. Expression of the open reading frame 74 (G-protein-coupled receptor) gene of Kaposi's sarcoma (KS)-associated herpesvirus: implications for KS pathogenesis. *J. Virol.* 73:6006-6014.

19. Larcher, F., R. Murillas, M. Bolontrade, C.J. Conti, and J.L. Jorcano. 1998. VEGF/VPF overexpression in skin of transgenic mice induces angiogenesis, vascular hyperpermeability and accelerated tumor development. *Oncogene*. 17:303–311.
20. Detmar, M., L.F. Brown, M.P. Schon, B.M. Elicker, P. Velasco, L. Richard, D. Fukumura, W. Monsky, K.P. Claffey, and R.K. Jain. 1998. Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J. Invest. Dermatol.* 111:1–6.
21. Gallo, R.C. 1998. The enigmas of Kaposi's sarcoma. *Science*. 282:1837–1839.
22. Greenblatt, R.M. 1998. Kaposi's sarcoma and human herpesvirus-8. *Infect. Dis. Clin. North Am.* 12:63–82.
23. Chor, P.J., and D.J. Santa Cruz. 1992. Kaposi's sarcoma. A clinicopathologic review and differential diagnosis. *J. Cutan. Pathol.* 19:6–20.
24. Niedt, G.W., P.L. Myskowski, C. Urmacher, D. Niedzwiecki, D. Chapman, S.E. Krown, and B. Safai. 1992. Histologic predictors of survival in acquired immunodeficiency syndrome-associated Kaposi's sarcoma. *Hum. Pathol.* 23:1419–1426.
25. Cornali, E., C. Zietz, R. Benelli, W. Weninger, L. Masiello, G. Breier, E. Tschachler, A. Albini, and M. Sturzl. 1996. Vascular endothelial growth factor regulates angiogenesis and vascular permeability in Kaposi's sarcoma. *Am. J. Pathol.* 149:1851–1869.
26. Nakamura, S., K. Murakami-Mori, N. Rao, H.A. Weich, and B. Rajeev. 1997. Vascular endothelial growth factor is a potent angiogenic factor in AIDS-associated Kaposi's sarcoma-derived spindle cells. *J. Immunol.* 158:4992–5001.
27. Masood, R., J. Cai, T. Zheng, D.L. Smith, Y. Naidu, and P.S. Gill. 1997. Vascular endothelial growth factor/vascular permeability factor is an autocrine growth factor for AIDS-Kaposi sarcoma. *Proc. Natl. Acad. Sci. USA.* 94:979–984.
28. Dupin, N., C. Fisher, P. Kellam, S. Ariad, M. Tulliez, N. Franck, E. Marck, D. Salmon, I. Gorin, J.P. Escande, et al. 1999. Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. *Proc. Natl. Acad. Sci. USA.* 96:4546–4551.
29. Shi, Q., S. Rafii, M.H. Wu, E.S. Wijelath, C. Yu, A. Ishida, Y. Fujita, S. Kothari, R. Mohle, L.R. Sauvage, et al. 1998. Evidence for circulating bone marrow-derived endothelial cells. *Blood.* 92:362–367.
30. Flore, O., S. Rafii, S. Ely, J.J. O'Leary, E.M. Hyjek, and E. Cesarman. 1998. Transformation of primary human endothelial cells by Kaposi's sarcoma-associated herpesvirus. *Nature.* 394:588–592.
31. Sun, R., S.F. Lin, L. Gradoville, Y. Yuan, F. Zhu, and G. Miller. 1998. A viral gene that activates lytic cycle expression of Kaposi's sarcoma-associated herpesvirus. *Proc. Natl. Acad. Sci. USA.* 95:10866–10871.