The Journal of Microbiology (2011) Vol. 49, No. 2, pp. 299-304
DOI 10.1007/s12275-011-0408-7

NOTE

Kaposi's Sarcoma-Associated Herpesvirus Infection of Endothelial Progenitor Cells Impairs Angiogenic Activity *In Vitro*

Seungchul Yoo[1], Sil Kim[1], Seungmin Yoo[1], In-Taek Hwang[2], Haewol Cho[1], and Myung-Shin Lee[1]*

[1]Department of Microbiology and Immunology, [2]Department of Obstetrics and Gynecology, Eulji University School of Medicine, Daejeon 301-746, Republic of Korea

(Received October 6, 2010 / Accepted November 29, 2010)

A recent study reported that endothelial progenitor cells (EPCs) are one of the reservoirs of Kaposi's sarcoma associated herpesvirus (KSHV). Although EPCs are closely linked to angiogenesis and vasculogenesis, little is known about the angiogenic potential of KSHV in EPCs. In this study, we used EPCs isolated from human umbilical cord blood to show that early infection by KSHV *in vitro* impaired the neovascularization of EPCs in matrigel. Our results suggest that KSHV may disrupt the angiogenic potential of EPCs and that the disseminated infection of KSHV could be associated with EPC dysfunction.

***Keywords*:** Kaposi's sarcoma-associated herpesvirus, endothelial progenitor cells, angiogenesis

Kaposi's sarcoma-associated herpesvirus (KSHV) is an etiologic agent for Kaposi's sarcoma (KS), an angioproliferative, tumor-like lesion (Pyakurel *et al.*, 2007). Endothelial cells are considered to be the precursors of KS spindle cells (Moses *et al.*, 1999), and reports have indicated that endothelial cells infected with KSHV show increased angiogenesis by production of several pro-angiogenic cytokines (Kang *et al.*, 2008). Additionally, KSHV gene-transfected mouse bone marrow endothelial lineage cells form vascular tumor like lesions (Mutlu *et al.*, 2007; Wang and Damania, 2008). Moreover, murine gammaherpesvirus 68 (MHV-68) infection of the apolipoprotein E-deficient mouse accelerated atherosclerosis, and a retrospective analysis of HIV-positive patients supported the association between KSHV and atherosclerosis (Alber *et al.*, 2000; Grahame-Clarke *et al.*, 2001). These studies imply that KSHV is closely linked to endothelial cells with respect to its pathogenesis.

Endothelial progenitor cells (EPCs), discovered in peripheral blood, contribute to postnatal vasculogenesis in tissue ischemia, wound healing, and tumor growth (Asahara *et al.*, 1997, 1999), although being a heterogeneous population, it is hard to define them accurately (Urbich, 2004). Endothelial colony forming cells (ECFCs), isolated from peripheral and umbilical cord blood, are characterized by outgrowth potential and vasculogenesis *in vivo*, and have been described as EPCs (Ingram *et al.*, 2004; Mead *et al.*, 2008).

Since EPCs are potential reservoirs of KSHV infection (Della Bella *et al.*, 2008) and can repair vessel damage and promote neovascularization (Asahara *et al.*, 1999), their functions could be affected by the angiogenic potential of KSHV. In this study, we provide the first report of the effects of KSHV on angiogenesis in ECFCs. Surprisingly, KSHV-infected ECFCs showed impairment, rather than the maintenance of, or increase in, angiogenesis *in vitro*. As impaired angiogenic activity of EPCs may promote endothelial dysfunction and cardiovascular disease (Werner *et al.*, 2005; Mund *et al.*, 2009), our results raise the possibility that KSHV may play a role in the development of cardiovascular disease such as atherosclerosis in KSHV-infected patients.

ECFCs and human umbilical cord vein endothelial cells (HUVECs) were isolated from umbilical cord blood and umbilical cord, respectively and cultured as described previously with slight modifications (Ingram *et al.*, 2004; Kestendjieva *et al.*, 2008). Umbilical cord blood samples (30 to 50 ml) and umbilical cords were collected from three healthy newborns (two boys, one girl; gestational age range, 36 to 40 weeks). All protocols were approved by the Institutional Review Board at the Eulji University School of Medicine, and informed consent was obtained from all parents. Umbilical cord blood was diluted 1:1 with phosphate buffered saline and was overlaid onto a half volume of Ficoll Hypaque (Sigma Aldrich, USA) and centrifuged continuously for 30 min at 740×g. Buffy coat cells were isolated and washed three times with endothelial growth medium (EGM-2; Cambrex, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 2% penicillin/streptomycin (Invitrogen, USA) (Complete EGM-2). Mononuclear cells, collected from 30 ml of umbilical cord blood, were seeded onto a six-well tissue culture plate (SPL life sciences, Korea) precoated with type 1 rat-tail collagen (BD Biosciences, USA) at 37°C, 5% CO_2, in a humidified incubator. Complete EGM-2 was changed every 24 h for seven days and then every other day until colony isolation. Colonies were released from the tissue culture plates and replated onto 60-mm culture dishes (SPL Life Sciences, Korea) coated with type 1 rat-tail collagen. HUVECs were isolated and cultured

* For correspondence. E-mail: mslee@eulji.ac.kr; Tel: +82-42-259-1662; Fax: +82-42-259-1669

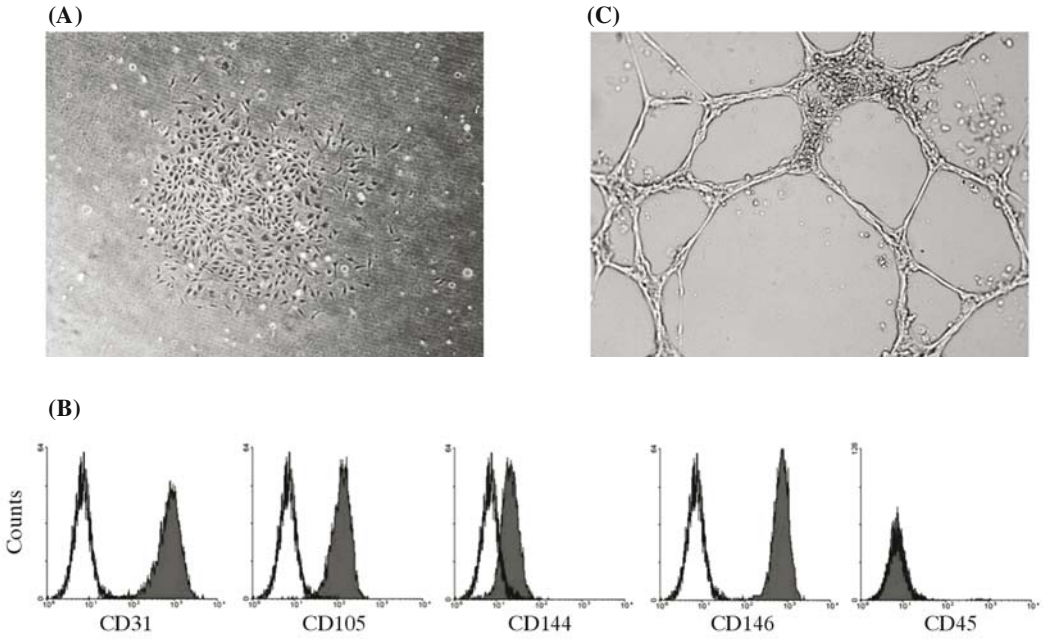

Fig. 1. Isolation of ECFCs from human umbilical cord blood. ECFCs were isolated from umbilical cord blood and cultured in complete EGM-2 medium, and HUVECs were isolated from the umbilical cord of the same donor for comparison. (A) Inverted phase-contrast microscopy showing a representative ECFC colony (day five, original magnification ×40, Nikon Eclipse TE2000-U, Nikon digital camera DXM1200F, Nikon ACT-1 software). ECFCs were cultured from mononuclear cells of umbilical cord blood as described. (B) Immunophenotyping of cell monolayers derived by flow cytometric analysis. ECFCs express CD31, CD105, CD144 and CD146, but do not express CD45. Representative data from three independent experiments with similar results using ECFCs from three different donors. Isotype controls are overlaid in white on each histogram for each surface antigen tested. (C) ECFCs were plated in matrigel for the formation of tube-like structures (original magnification ×40). The images are representative of three independent experiments using different ECFCs with similar results.

from umbilical cord as described previously, except that we used EGM-2 with growth supplements for culture (Kestendjieva *et al.*, 2008). ECFCs and HUVECs were identified by flow cytometry (BD FACS Calibur™) using various cell surface-specific FITC-conjugated antibodies. Cell viability was analyzed with the cell proliferation reagent WST-1 (Roche Applied Sciences, USA) according to the manufacturer's instructions. BCBL-1 and 293 cells harboring a recombinant KSHV, BAC36,

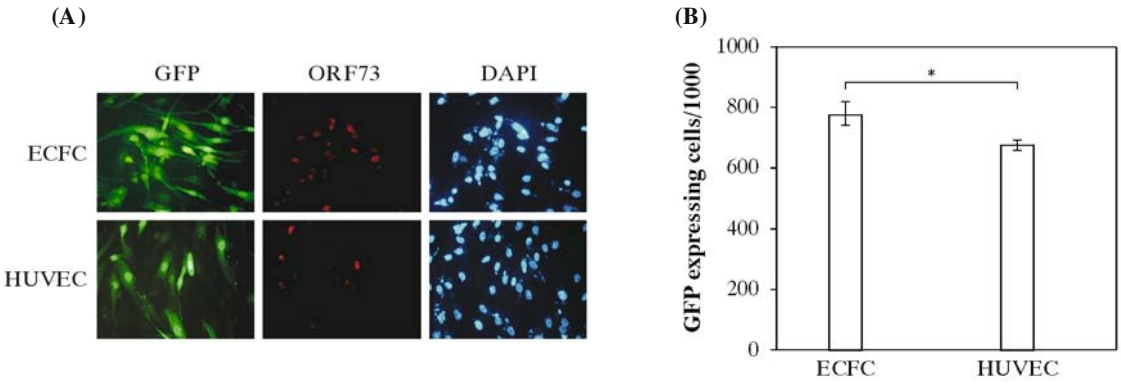

Fig. 2. KSHV infection on ECFCs. (A) After infection, KSHV BAC36-infected cells were fixed, permeabilized, and incubated with primary rat mAb directed against KSHV ORF73. Cells were washed with PBS, followed by incubation with rhodamine-conjugated goat anti-rat immunoglobulin. KSHV ORF73 staining is represented by red. DAPI stain (blue) shows the nuclei. Representative results of three independent experiments are shown. Cells were visualized through fluorescence microscope with 100× magnification. (B) ECFCs and HUVECs isolated from the same donors were tested for KSHV infection. Since KSHV BAC36 contains a GFP cassette, KSHV infectivity was evaluated by counting the number of GFP-positive cells per 1,000 cells. These assays were performed in triplicate and the mean values are shown (*$p<0.05$, ECFC vs. HUVEC).

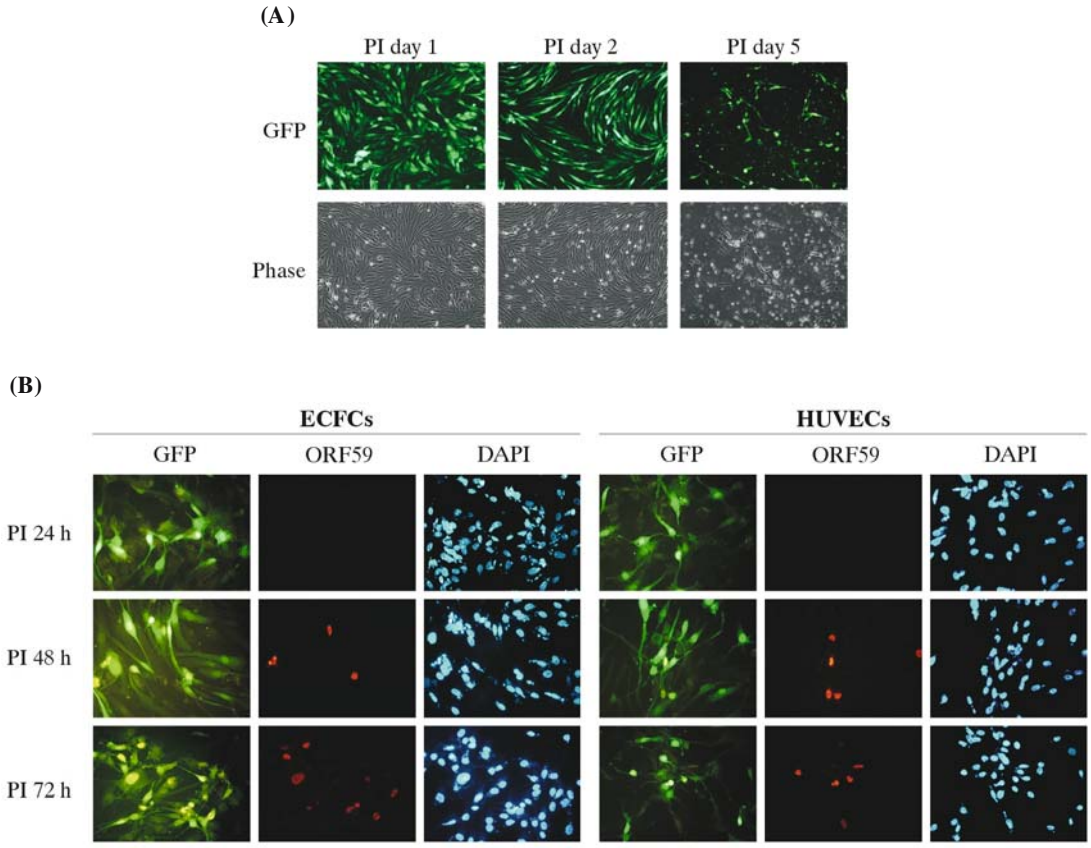

Fig. 3. Lytic replication of KSHV-infected ECFCs. (A) KSHV-infected ECFCs were observed at one, two and five days postinfection. Inverted fluorescence microscopy (original magnification ×100) showing KSHV-infected cells expressing GFP. Morphological change into spindle shape was observed between one day and two days postinfection. Cell death was initiated at around three days postinfection and only a small population of cells remained at day five. (B) Expressions of KSHV lytic cycle protein were observed in ECFCs and HUVECs. KSHV BAC36-infected cells were fixed, permeabilized, and incubated with primary mouse mAbs directed against KSHV ORF59. Cells were washed with PBS, followed by incubation with rhodamine-conjugated goat anti-mouse IgG. KSHV ORF59 staining is represented by red. DAPI stain (blue) shows the nuclei. Representative results of three independent experiments are shown. Cells were visualized through fluorescence microscope with 100× magnification.

have been described previously (Zhou *et al.*, 2002; Gao *et al.*, 2003). KSHV BAC36, containing a green fluorescent protein (GFP) cassette, was induced by treating 20 ng/ml of tetradecanoyl phorbol acetate (TPA, Sigma-Aldrich) and the isolated virus was used to infect HUVECs as described previously (Yoo *et al.*, 2008). Infection was conducted by centrifugation at 2,000×g for 60 min with 5 μg/ml of polybrene (Sigma Aldrich). Infectivity was estimated by examining GFP expression under an inverted fluorescence microscope. Immunofluorescence assay was conducted to analyze the expression of viral proteins. Briefly, cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.3% Triton X-100, and blocked with 5% bovine serum albumin. Cells were incubated with anti-ORF73 (Advanced Biotechnologies Inc., USA, 1:250) or anti-ORF59 (Advanced Biotechnologies Inc., 1:250) overnight at 4°C followed by incubation with rhodamine-labeled anti-rat antibody (KPL, UK) or rhodamine-labeled anti-mouse antibody (KPL) for 30 min at room temperature. After washing, sample was mounted with Vectashield® (Vector laboratories, Inc, USA) with 4,6-diamidino-2-phenylindole (DAPI). Analyses were performed using a Nikon ECLIPSE E400 fluorescence microscope. To investigate the activity of capillary like tube formation *in vitro*, the Lab-Tek® chamber slide™ system (Sigma Aldrich) was coated with a matrigel matrix basement membrane (BD Biosciences). KSHV BAC36- or PBS mock-infected cells were grown for 24 h and approximately 1×10^6 cells were harvested from each in complete EGM-2 and seeded onto matrigel- coated wells. Images of cellular network structures were captured three hours into the incubation period. We chose a random field of view (FOV) at ×40 magnification per treatment, and counted the average number of branches per FOV.

ECFCs were isolated from samples of umbilical cord blood from three donors and HUVECs were simultaneously isolated from the umbilical cords of the same donors for comparison. ECFC colonies from umbilical cord blood appeared between four and 13 days of culture. Since some mesenchymal stem cell colonies were also present, we isolated only those cells showing a cobblestone appearance and expressing the EC surface antigens CD31, CD105, CD144, and CD146, but not the

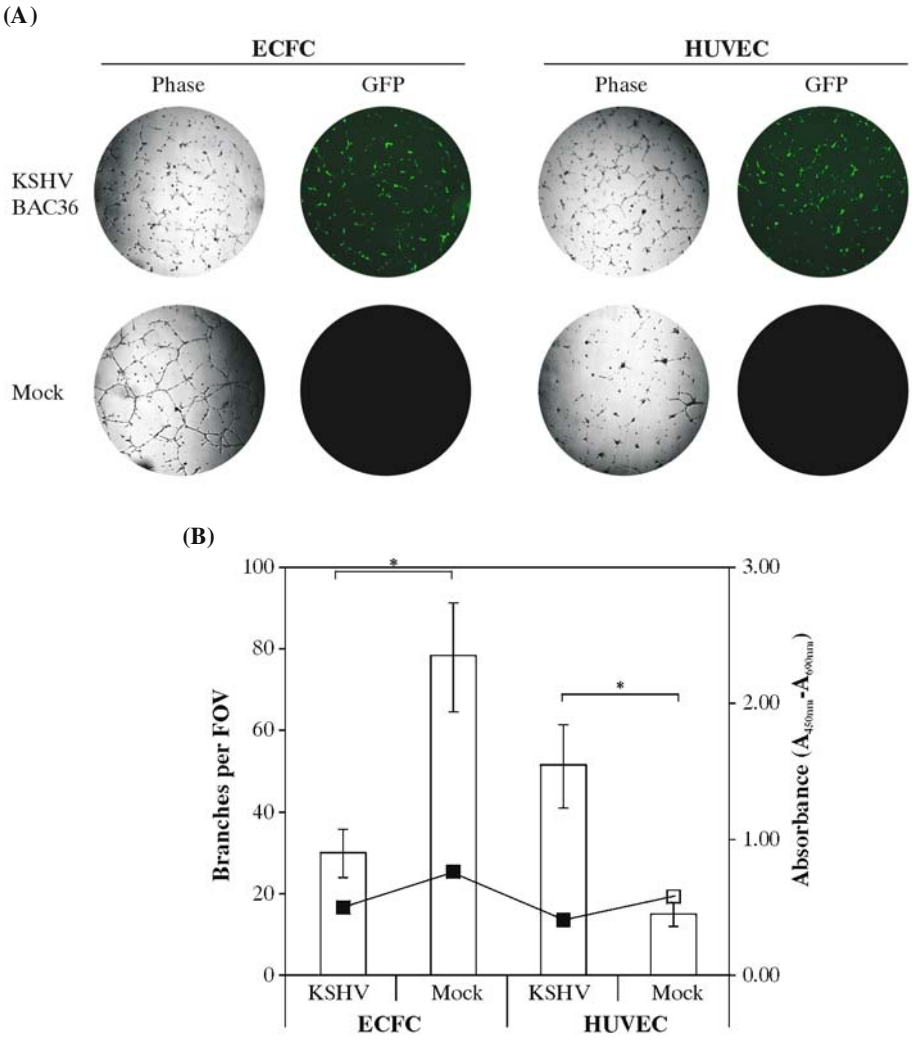

Fig. 4. KSHV infection in ECFCs impairs angiogenesis *in vitro.* (A) Tube formation of KSHV-infected ECFCs and HUVECs was investigated in matrigel. Cells were infected with KSHV for 24 h and the same number of infected cells was used in the matrigel assay. Tube formation and GFP expression were observed under inverted fluorescence microscopy after three hours of incubation on matrigel (original magnification ×40). The images are representative of three independent experiments using different ECFCs with similar results. (B) Angiogenic index *in vitro* and cell proliferation rate of KSHV- and mock-infected cells. The average number of branches per field of view (FOV) was determined for each experimental group. Cell proliferation was analyzed using the WST-1 reagent and then determined by reading absorbances. Bars represent branches per FOV and black squares the absorbance measured in the cell proliferation assay. The data was obtained from experiments performed in triplicate and the mean values are shown (*$p<0.05$).

hematopoietic cell surface marker CD45 (Figs. 1A and B). The isolated cells also formed capillary-like structures on matrigel (Fig. 1C). These cells were confirmed as being endothelial in origin as they had characteristic endothelial features under defined ECFC culture conditions. For comparison of characteristics after KSHV infection, we isolated HUVECs from the same donors and confirmed their expression of EC surface antigens (data not shown). After 24 h after infection, GFP and ORF73 of KSHV were expressed in infected ECFCs and HUVECs (Fig. 2A). KSHV BAC36 infectivity was monitored by measuring GFP expression (Zhou *et al.*, 2002). KSHV infectivity of ECFCs was slightly higher than that of HUVECs (Fig. 2B). After infection, we investigated the changes in GFP expression and the fate of infected cells. Initially, about 90% of cells expressed GFP and infected cells gradually became spindle-shaped between 24 and 48 h. After 72 h, infected cells began to die, and only a very small population of infected cells remained at five days postinfection (Fig. 3A). KSHV BAC36 infection of HUVECs is known to induce lytic replication from around three days postinfection (Gao *et al.*, 2003). KSHV-infected ECFCs showed similar response to lytic replication as KSHV-infected HUVECs. The expression of ORF59, one of the proteins expressed in lytic replication, could be observed in ECFCs and HUVECs from 48 h after infection (Fig. 3B). This result indicates that EPCs support the lytic replication of KSHV and cytopathogenic effect can be induced, which is consistent with previously published study (Della Bella *et al.*, 2008).

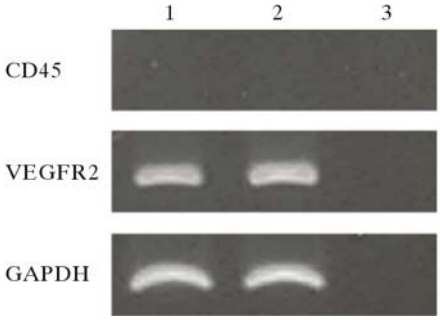

Fig. 5. RT-PCR analysis of KSHV- or mock-infected ECFCs for gene expression of CD45, VEGFR-2 and GAPDH. At 24 h post infection, total RNA was collected from KSHV- or mock-infected HUVECs, and mRNA expression was analyzed by RT-PCR. Lanes 1, 2, and 3 show the RT-PCR products from KSHV-infected ECFCs, mock-infected ECFCs and the non-template control, respectively. Results are representative of 3 independent experiments.

To investigate the activity of tube formation *in vitro*, we seeded the same number of KSHV- and mock-infected cells onto matrigel at 24 h postinfection. As complete tube-like structures appeared within 2 h after seeding of mock-infected ECFCs, we captured the images and analyzed the tube formation three hours after seeding. At that time, mock-infected HUVECs formed far fewer tube-like structures on matrigel than ECFCs (Fig. 4A). This result suggests that the angiogenic activity of ECFCs *in vitro* is much greater than that of HUVECs. KSHV-infected HUVECs showed increased tube formation compared with mock-infected HUVECs, which is consistent with previous studies (Kang *et al.*, 2008). Surprisingly, tube formation was impaired in KSHV-infected ECFCs (Figs. 4A and B bar graph). Since this result might be due to differences in proliferation rate or cell death rate, we investigated the proliferation rate between 24 and 48 h, and found that it decreased in KSHV-infected cells compared to mock-infected cells. However, as the proliferation of both KSHV-infected ECFCs and HUVECs was suppressed at a similar rate, the impaired angiogenic activity of KSHV-infected ECFCs cannot be explained by an impaired proliferation rate or cell death (Fig. 4B, black square). To exclude the possibility of contamination by other mononuclear cells in blood, CD45 and VEGFR-2 mRNA expression was analyzed by RT-PCR after KSHV infection. The results (Fig. 5) revealed that there was no detectable change in mRNA expression after KSHV infection and no CD45 mRNA expression in KSHV- or mock-infected ECFCs, indicating that the KSHV-infected cells were free of mononuclear cell contamination. In a previous study, it was reported that EPCs from KS patients could form capillaries as efficiently as control endothelial cells, which is contradictory to the results presented in this study. Although the exact mechanism of the suppressive effect of KSHV infection in ECFCs was not investigated in detail in this study, one possibility is that the contradiction is due to differences in susceptibility to lytic replication. As ECFCs had a higher proliferation rate than HUVECs, KSHV-infected cells might have a different reponse to lytic replication. Although ORF59 protein expression was detected at 48 h post-infection in HUVECs and ECFCs, orf59 mRNA expression was reported to occur as early as 16 h post-infection in HUVECs using this system. Therefore, an early phase of lytic gene expression might involve aniogenesis, although its effects on proliferation would be limited. In addition, as the status of most EPCs from KS patients was latent replication, there might be some differences between angiogenesis in this study and that in the previous report. Another possibility is the expression of angiogenic or inflammatory cytokines. Induction of pro-angiogenic and pro-inflammatory cytokines, such as IL-6, Ang-2, MMP-1, MMP-2, and MMP-9, has been described in KSHV-infected HUVECs (Qian *et al.*, 2007; Kang *et al.*, 2008). Some of these cytokines might be expressed differently in KSHV-infected ECFCs, which might down-regulate the angiogenesis of ECFCs. However, as cytokines elicit complex effects *in vivo*, further intensive studies to elucidate there actions will be required. This is the first report that viral infection of endothelial progenitor cells can impair their angiogenic activity. In a previous report, MHV-68 accelerated atherosclerosis in the apolipoprotein E-deficient mouse (Alber *et al.*, 2000). There have also been reports that HIV infection and KSHV infection are correlated with a risk of cardiovascular disease, such as atherosclerosis (Grahame-Clarke *et al.*, 2001; Carletti *et al.*, 2002; Sklar and Masur, 2003; Thiébaut *et al.*, 2005; Martin *et al.*, 2006). Another report suggests that KSHV infection in humans can disseminate to EPCs (Della Bella *et al.*, 2008). The infection of a significant proportion of ECFCs with KSHV under conditions that exaggerate infection, such as immunosuppression, would impair their angiogenic activities and promote endothelial cell dysfunction, and may be one explanation for the increased incidence of cardiovascular disease in HIV- and KSHV-infected patients.

We thank Jamie Case and David A. Ingram, Indiana University School of Medicine, for help in isolating ECFCs. This study was supported by a Korea Research Foundation (KRF) grant funded by the Korea government (MEST) (No. 2009-0069011).

References

Alber, D., K. Powell, and P. Vallance. 2000. Herpesvirus infection accelerates atherosclerosis in the apolipoprotein E-deficient mouse. *Circulation* 15, 779-785.

Asahara, T., H. Masuda, T. Takahashi, C. Kalka, C. Pastore, M. Silver, M. Kearne, M. Magner, and J.M. Isner. 1999. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ. Res.* 85, 221-228.

Asahara, T., T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, and J.M. Isner. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275, 964-967.

Carletti, F., C. Mandolini, A. Rossi, M.R. Capobianchi, and M.C. Borgia. 2002. Prevalence of human herpesvirus (HHV)-8 infection among carriers of cardiovascular disease. *J. Biol. Regul. Homeost. Agents* 16, 110-113.

Della Bella, S., A. Taddeo, M.L. Calabrò, L. Brambilla, M. Bellinvia, E. Bergamo, M. Clerici, and M.L. Villa. 2008. Peripheral blood endothelial progenitors as potential reservoirs of Kaposi's sarcoma-associated herpesvirus. *PLoS ONE* 3, e1520.

Gao, S.J., J.H. Deng, and F.C. Zhou. 2003. Productive lytic replication of a recombinant Kaposi's sarcoma-associated herpesvirus in efficient primary infection of primary human endothelial cells.

J. Virol. 77, 9738-9749.

Grahame-Clarke, C., D. Alber, and S. Lucas. 2001. Association between Kaposi's sarcoma and atherosclerosis: implications for gammaherpesviruses and vascular disease. *AIDS* 15, 1902-1904.

Ingram, D.A., L.E. Mead, H. Tanaka, V. Meade, A. Fenoglio, K. Mortell, K. Pollok, M.J. Ferkowicz, D. Gilley, and M.C. Yoder. 2004. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* 104, 2752-2760.

Kang, T., F.C. Ye, S.J. Gao, and L.D. Wang. 2008. Angiogenesis, kaposi's sarcoma and kaposi's sarcoma-associated herpesvirus. *Virol Sin.* 23, 449-458.

Kestendjieva, S., D. Kyurkchiev, G. Tsvetkova, T. Mehandjiev, A. Dimitrov, A. Nikolov, and S. Kyurkchiev. 2008. Characterization of mesenchymal stem cells isolated from the human umbilical cord. *Cell Biol. Int.* 32, 724-732.

Martin, L., O. Vandhuick, P. Guillo, and V. Bellein. 2006. Premature atherosclerosis in HIV positive patients and cumulated time of exposure to antiretroviral therapy (Shiva study). *Atherosclerosis* 185, 361-367.

Mead, L.E., D. Prater, M.C. Yoder, and D.A. Ingram. 2008. Isolation and characterization of endothelial progenitor cells from human blood. *Curr. Protoc. Stem Cell Biol.* Chapter 2, Unit 2C 1.

Moses, A.V., K.N. Fish, R. Ruhl, P.P. Smith, J.G. Strussenberg, L. Zhu, B. Chandran, and J.A. Nelson. 1999. Long-term infection and transformation of dermal microvascular endothelial cells by human herpesvirus 8. *J. Virol.* 73, 6892-6902.

Mund, J.A., D.A. Ingram, M.C. Yoder, and J. Case. 2009. Endothelial progenitor cells and cardiovascular cell-based therapies. *Cytotherapy* 11, 103-113.

Mutlu, A.D.A., L.E. Cavallin, L. Vincent, C. Chiozzini, P. Eroles, E.M. Duran, Z. Asgari, and *et al.* 2007. *In vivo*-restricted and reversible malignancy induced by human herpesvirus-8 KSHV: a cell and animal model of virally induced Kaposi's sarcoma. *Cancer Cell.* 11, 245-258.

Pyakurel, P., F. Pak, A. Mwakigonja, and E. Kaaya. 2007. KSHV/HHV-8 and HIV infection in Kaposi's sarcoma development. *Infect. Agents Cancer* 2, 4.

Qian, L.W., J. Xie, F. Ye, and S.J. Gao. 2007. Kaposi's sarcoma-associated herpesvirus infection promotes invasion of primary human umbilical vein endothelial cells by inducing matrix metalloproteinases. *J. Virol.* 81, 7001-7010.

Sklar, P. and H. Masur. 2003. HIV infection and cardiovascular disease-is there really a link? *N Engl. J. Med.* 349, 2065-2067.

Thiébaut, R., V. Aurillac-Lavignolle, and F. Bonnet. 2005. Change in atherosclerosis progression in HIV-infected patients: ANRS Aquitaine Cohort, 1999-2004. *AIDS* 19, 729-731.

Urbich, C. 2004. Endothelial progenitor cells: Characterization and role in vascular biology. *Circ. Res.* 95, 343-353.

Wang, L. and B. Damania. 2008. Kaposi's sarcoma-associated herpesvirus confers a survival advantage to endothelial cells. *Cancer Res.* 68, 4640-4648.

Werner, N., S. Kosiol, T. Schiegl, P. Ahlers, K. Walenta, A. Link, M. Bohm, and G. Nickenig. 2005. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl. J. Med.* 353, 999-1007.

Yoo, S.M., A.K. Ahn, T. Seo, H.B. Hong, M.A. Chung, S.D. Jung, H. Cho, and M.S. Lee. 2008. Centrifugal enhancement of Kaposi's sarcoma-associated virus infection of human endothelial cells *in vitro*. *J. Virol Methods* 154, 160-166.

Zhou, F.C., Y.J. Zhang, J.H. Deng, X.P. Wang, H.Y. Pan, E. Hettler, and S.J. Gao. 2002. Efficient infection by a recombinant Kaposi's sarcoma-associated herpesvirus cloned in a bacterial artificial chromosome: application for genetic analysis. *J. Virol.* 76, 6185-6196.