U94 of human herpesvirus 6 inhibits in vitro angiogenesis and lymphangiogenesis

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Human herpesvirus 6 (HHV-6) is a lymphotropic virus, but recent observations showed that also vascular endothelial cells (ECs) are susceptible to infection, both in vivo and in vitro. The observation that lymph nodes are a site of viral persistence suggests that lymphatic ECs (LECs) might be even more relevant for HHV-6 biology than vascular ECs. Here, we provide evidence that HHV-6 can infect LECs in vitro and establish a latent infection. Thus HHV-6 infection induces the loss of angiogenic properties both in LECs and in vascular ECs, as shown by the inability to form capillary-like structures and to seal wound scratches. The antiangiogenic effects observed in infected cells are associated to the expression of HHV-6 U94/rep, a latency-associated gene. In fact, transfection of U94/rep or addition of recombinant U94/REP protein to ECs inhibits the formation of in vitro capillary-like structures, reduces migration of ECs, and blocks angiogenesis, rendering rat aortic rings insensitive to VEGF-induced vasculogenic activity. The ability of U94/rep to block different angiogenic steps may lead to approaches in the potential control of the proliferation of blood and lymphatic vessels.

endothelial cells | lymphatic endothelial cells

Human herpesvirus type 6 (HHV-6) was first isolated in 1986 (1). HHV-6 is characterized by an elective tropism for T-lymphocytes and macrophages, but the virus can infect several different cells. The cell entry receptor is CD46, a glycoprotein expressed on the surface of all human cells, and virus host range is not limited to lymphatic tissue, as shown by in vivo detection of viral DNA in several organs, including liver, thyroid, and skin. Viral strains cluster in two variants, designated HHV-6A and HHV-6B, closely related by DNA homology but different with respect to in vitro cell host range, reactivity with monoclonal antibodies, and, most likely, also for pathogenic properties (2). HHV-6B is the etiologic agent of Exanthem Subitum, a childhood benign febrile disease, and HHV-6A has not yet been linked to specific human pathologies. Other diseases have been proposed to be associated to HHV-6 infection (i.e., multiple sclerosis and chronic fatigue syndrome) but no causal link so far has been firmly established. After primary infection, occurring in the early childhood, HHV-6 establishes a latent infection and resides mainly in peripheral blood mononuclear cells (PBMCs) (3) and in macrophages (4). In fact, small amounts of viral DNA are commonly detected in PBMCs of healthy adults in the absence of viral replication, with prevalence >80% (5). Approximately 5% of the healthy adult population has anti-HHV-6 IgM antibodies, suggesting that virus reactivation occurs in the absence of clinical symptoms (6). Reactivation in immunocompromised and transplanted patients may be associated with severe conditions, such as encephalitis, pneumonitis, and graft failure (2). The molecular mechanism of latency and reactivation are still unknown. Kondo et al. reported that specific transcripts from the immediate early region of viral DNA are expressed during latency, similarly to the situation previously described for human cytomegalovirus (7). However, during latency also the transcript from the viral gene U94/rep is detected (8). U94/rep

has no homologue among other human herpesviruses, but it has a strong homology with the adeno-associated parvovirus Rep 68/78 gene (9), a pleiotropic effector of virus integration, replication, and transcription. The function of HHV-6 U94/rep is unknown, but the high conservation of the gene all HHV-6 strains implies that it has a vital role in virus biology (10). U94/REP has single stranded DNA binding activity, might be involved in DNA replication and is expressed at low levels during the early phases of viral replication (10). We proposed that expression of U94/rep can be considered a molecular marker of viral latency, since its mRNA is detected in PBMCs from latently infected healthy individuals in the absence of other viral lytic transcripts (8). We have also shown that U94/REP is a negative regulator of viral replication, and T-cell lines stably expressing U94/rep do not support productive viral replication (8). Purified U94/REP protein, exogenously produced in bacterial systems, blocks selectively the replication of beta-herpesviruses in infected cell cultures, without showing any effect on alpha- and gamma-herpesviruses (11). Recently, HHV-6 DNA sequences were detected in vascular blood endothelial cells (ECs), and in vitro experiments provided evidence that vascular ECs are important targets for HHV-6 infection (12–15). In particular, vascular ECs are susceptible to infection both in vitro and in vivo, forming a site where the virus can persist in the absence of cytopathic effect. Virus infection has a significant effect on EC physiology, upregulating the production of specific chemokines, such as MCP-1, IL-8, and RANTES (14, 15). The observations that lymph nodes are a site of viral persistence and viral infection has been associated to lymphadenitis (16) suggest that lymphatic ECs (LECs) from lymphatic tissue might be even more relevant for HHV-6 biology than vascular ECs. However, no information is available on the ability of HHV-6 to infect LECs, due to the lack of suitable in vitro systems. Here, we provide evidence that HHV-6 infects LECs, establishing a latent infection. More interestingly, we describe that HHV-6 infection, both in LECs and in vascular ECs, results in a strong inhibition of angiogenesis, and that this effect is entirely due to the expression of U94/rep.

Results

HHV-6 Infection of LECs. LECs, purified, and cultured, as previously described (17), were infected with cell-free inocula of HHV-6A. Morphology of infected cultures was similar to control LECs and no cytopathic effect was detected. HHV-6 infected LECs maintained a doubling time of 4–6 days, similar to uninfected LECs, showing that HHV-6 infection does not mod-

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Fig. 1. Replication of HHV-6 in LECs. LECs were infected with a cell-free HHV-6 inoculum, then cell pellets, and the corresponding supernatants were collected at the indicated time points. HHV-6 DNA was quantified by real time PCR (A). Results are shown as mean $\pm$ SD referred to duplicate samples of three independent experiments. HHV-6 transcription was analyzed by single round (B) and nested RT-PCR (C), using total RNA extracted from infected LECs at the indicated time points. RNA was retrotranscribed and amplified with primers specific for U22 (late gene), U42 (IE gene), and U94 (latency-associated gene). Amplification of β -actin is shown as positive control.

ify the cell cycle. Immunocytochemical analysis with mAb to HHV-6 gp60/110 showed only few productively infected cells, and $\approx 1\text{--}2\%$ LECs were positive at day 7 after viral infection. No positive signal was detected at day 14 p.i.. Real-time quantitation of viral DNA extracted from infected cell cultures showed the presence of viral DNA at all time points. The amount of intracellular HHV-6 DNA ranged between 5.8×10^7 and 6×10^6 genome equivalents in 10^6 cells, up to 21 days p.i. (Fig. 1A). Extracellular viral DNA decreased from 10^6 genomes/mL of medium at 3 days p.i. to 2×10^2 genomes/mL at 21 days p.i. (Fig. 1A). To determine the replicative state of HHV-6 in infected LECs, viral transcription was analyzed by RT-PCR for the presence of immediate-early (U42) and late (U22) viral lytic transcripts, and for U94/*rep*, the latency-associated transcript that is expressed at low levels, also during productive infection. PCR reactions had sensitivities similar for all genes, detecting 1,000 target molecules after first round PCR and 50–100 target molecules after nested PCR. The transcripts of U42 and U22 were detected by first round PCR only during the first 3 days p.i. (Fig. 1B), and were present until 14 days p.i. after nested PCR (Fig. 1C). Interestingly, the samples were positive to U94/*rep* mRNA after first round PCR at all time points, suggesting that this transcript is constantly transcribed, even when lytic genes are not present, or expressed at low levels detectable only by nested PCR (Fig. 1B and C). It is relevant to mention that U94/*rep* transcripts in lytically infected lymphoid cells are significantly less abundant than the transcripts of lytic genes. In fact, during lytic infection of JJhan lymphoid T cells the transcripts of immediate early (U42) and late (U22) genes are ≈ 100 -fold more abundant than U94/*rep*, in agreement with the original description by Rapp et al. (10). These results suggest that productive infection took place only for a few days, and that afterward HHV-6 genomes persisted in a latent state, as shown by the persistence of U94/*rep* mRNA and by the steady amount of intracellular viral DNA. To confirm that LECs support productive viral replication, target lymphoid T cells were cocultured with LECs infected with HHV-6A or 6B. In additional experiments, target cells were infected with culture medium concentrated from infected LECs. Viral infection of target cells was analyzed by real-time PCR and IFA specific for HHV-6 gp116. The results of both assays show that HHV-6-infected LECs produce infectious virus. In fact, target cells were productively infected, as shown by the appearance of HHV-6 typical cytopathic effect (Fig. S1A), by positivity to HHV6 lytic antigens in IFA (Fig. S1B and C) and by increasing amount of viral DNA, reaching up to 10^9 genome copies in 15,000 cells. Similar results were obtained when LECs were infected with HHV-6B.

HHV-6 Infection Inhibits the Formation of Capillary-Like Structures. To evaluate the effect of HHV-6 infection on LECs physiological properties relating to angiogenesis, we assayed their ability to form

capillary-like structures on basal membrane extracts (Cultrex® BME). When LECs are cultured on BME in the presence of VEGF-C, they spontaneously form hollow tube-like structures (17). As shown in Fig. 2*A* and *C*, mock-infected LECs, harvested 3 or 7 days postinfection, formed a wide array of capillary-like structures. Instead, HHV-6 infected LECs were unable to form tubes. The inhibition of tube formation was present as early as 3 days p.i. (Fig. 2*B*), and was particularly pronounced 7 days p.i. when almost no tube-like structures were formed (Fig. 2*D*). Similar results were obtained infecting LECs with HHV-6A (Fig. 2) or HHV-6B (Fig. S2).

Expression of HHV-6 U94/*rep* Inhibits In Vitro Capillary Formation. The highest degree of inhibition of LECs angiogenesis was observed 7 days p.i., when the latency-associated U94/*rep* HHV-6 transcript was prevalent over lytic genes. To test the hypothesis that the block of in vitro capillary formation might be mediated by U94/*rep*, we transfected plasmid pSR2pH, expressing the full length form of U94/*rep*, into LECs. As previously described, this plasmid expresses a biologically active U94/REP protein (11). To ensure that the transfected sequences were functional, cells harvested at different time points after transfection were analyzed for the presence of U94/*rep* mRNA. U94/*rep* gene was constantly transcribed, and even 5 days after transfection cells were still positive for U94/*rep* mRNA (Fig. 3A). LECs, transfected with pSR2pH or with pSR2 (the same vector carrying no insert), were harvested 2 days after transfection and transferred

Fig. 2. Effects of HHV-6 infection on organization of LECs on BME. HHV-6 infection inhibited LECs organization into capillary-like networks. The inhibitory effect was partial 3 days post infection (d.p.i.) (B) and total 7 days p.i. (D), whereas mock infected LECs spontaneously formed tubes both at day 3 (A) and day 7 (C). Pictures were taken after 6 h incubation at 37 °C (original magnification, $\times 10$). Data are representative of three independent experiments with similar results.

Fig. 3. Expression of HHV-6 U94/*rep* inhibits network formation on BME. The presence of U94/*rep* mRNA was analyzed by RT-PCR in HUVECs transfected with plasmid pSR2pH at different days post transfection (d.p.t.) (A). As a control, amplification of β -actin mRNA is also shown. Tube formation was inhibited by U94/*rep* expression (B). Cells transfected with control plasmid pSR2 (1, 3) or with U94/*rep* plasmid pSR2pH (2, 4), were transferred on BME-coated plates 48 h after nucleofection. Absence of the capillary-like network was evident both in LECs (2) and in HUVECs (4). Images were taken 6 h after plating (original magnification, $\times 10$). Data are representative of three independent experiments with similar results.

on BME-coated plates. LECs transfected with pSR2 maintained the ability to form a complex network of capillary-like structures (Fig. 3B), whereas pSR2pH-transfected cells exhibited an adherent, flattened morphology, and did not form tubes (Fig. 3C). To evaluate if this inhibition may be considered a general response of ECs we analyzed the effect of U94/rep-expressing plasmid also on HUVECs. Also in this case, pSR2-transfected HUVECs were able to form cords (Fig. 3D) and pSR2pH-transfected HUVECs adhered to BME and developed only few hollow tube-like structures (Fig. 3E). To verify the specificity of inhibition, HUVECs were transfected with plasmids carrying deleted forms of U94/rep. Both pSR2pH-ΔC and pSR2pH-ΔN, deleted respectively in the carboxy- and amino-terminal portions, did not affect the formation of cords (Fig. S3).

HHV-6-Encoded U94/REP Protein Inhibits In Vitro Capillary Formation.

Previously, we reported the production and purification of a functional U94/REP recombinant protein (11). Therefore, we performed experiments to determine if the U94/REP protein, added exogenously to ECs cultures, induces the same suppression of angiogenic potential observed after transfection of the U94/*rep* gene. Due to limited availability of LECs, and considering that transfection of U94/*rep* yields similar results in HUVECs and in LECs, all subsequent experiments were carried out only on HUVEC cells. Recombinant U94/REP was added to the culture medium of HUVECs at different concentrations (0.5, 1, and 2 $\mu\text{g/mL}$), and cell viability was assayed by MTT and propidium iodide staining. No significant toxicity or cell death was present in the cultures treated with recombinant U94/REP, confirming the results previously reported (11). The ability of the recombinant protein to be internalized by ECs was analyzed by IFA, using a polyclonal serum to a synthetic peptide derived from the U94/*rep* sequence. The results show that U94/REP collects at the cell membrane, is internalized as indicated by a bright generalized staining after 48 h, and accumulates in the cell nucleus after 72 h, suggesting a specific nuclear targeting of the protein (Fig. 4A). HUVECs treated with U94/REP for 72 h were transferred on BME-coated plates, and observed for the formation of capillary-like structures (Fig. 4B). HUVECs treated with

Fig. 4. Recombinant U94/REP inhibits network formation on BME. Uptake of recombinant U94/REP protein was analyzed by IFA in HUVECs (A). Cells, seeded on collagen-coated culture slides were treated with 2 $\mu\text{g}/\text{mL}$ of recombinant U94/REP for the indicated times. IFA assays were performed using a rabbit pAb directed against a U94/REP epitope. Images were taken with a UV light microscope (Nikon Eclipse E600) equipped with a digital camera (DMX 1200). Original magnification, $\times 100$. Tube forming assay was performed on HUVECs treated with different doses of recombinant U94/REP (B). Cells treated for 72 h in the presence or absence of the indicated amounts of protein, were harvested and transferred onto BME-coated wells. Untreated HUVECs formed capillary-like structures within 6 h after seeding. Treatment with U94/REP inhibited the differentiation of HUVECs into capillaries in a dose dependent manner. Data are representative of four independent experiments with similar results.

0.5 $\mu\text{g/mL}$ of U94/REP formed tube-like structures, but the network was significantly altered when compared to control cells. Exposure to 1 $\mu\text{g/mL}$ of U94/REP resulted in the formation of few capillary-like structures, and treatment with 2 $\mu\text{g/mL}$ of U94/REP resulted in the complete loss of ability to form tube-like structures.

HHV-6 U94/rep and Its Gene Product Affect Endothelial Cell Migration.

We studied also the effect of U94/*rep* on in vitro wound healing from mechanical injury. HUVECs were transfected with pSR2pH, harvested 24 h after transfection, and grown on collagen coated plates. Confluent monolayers were scratched with a 200- μ L tip, and the percentage of wound sealing was observed over a period of 10 h. As shown in Fig. 5 *A* and *B*, control HUVECs, transfected with the control plasmid pSR2, reached 80% sealing, whereas HUVECs transfected with U94/*rep* reached only 20–30% sealing (Fig. 5 *C* and *D*), showing a considerable impairment in scrape wound repair ability. To study the potential long term effect of U94/REP on the motility rate of ECs, we evaluated HUVECs' cell migration activity (18). HUVECs were treated with recombinant U94/REP (4 μ g/mL), and cell movement along the bottom of a tissue culture flask was recorded after 8 days. As shown in Fig. 5 *E* and *F*, cell migration decreased considerably in U94/REP treated cells.

U94/REP Blocks Angiogenesis of Rat Aortic Rings. The effect of U94/REP on angiogenesis was studied also using the aorta ring assay (19). This method permits to investigate the ability of molecules to interfere with the ex vivo growth of mammal microvessels. Treatment of aortic rings with U94/REP resulted in a sharp reduction of microvessel outgrowth (Fig. 6). Maximal inhibition of angiogenesis induced by U94/REP was observed after 7 days of incubation at 4 $\mu\text{g/mL}$. At this time, the number of microvessels in control cultures was 45 ± 18 compared to 5 ± 4 in rings treated with U94/REP. As expected, stimulation of aorta rings with 20 ng/mL of VEGF strongly increased microvessel outgrowth (285 ± 74). Addition of U94/REP to rings treated

Fig. 5. U94/REP reduces endothelial cell migration. In the wound healing assay, HUVECs were nucleofected with the control vector pSR2 (A and B) or with the U94/rep-expressing pSR2pH plasmid (C and D). Confluent monolayers were scratched using a 200- μ L pipette tip and serum was increased to 10% to facilitate cell migration. HUVEC migration was recorded by light microscopy over a 10 h time course after wound scratch. Representative images of wound sealing at 0 (A and C) and 10 h (B and D) after wound scratch are shown (original magnification, $\times 10$). In the cell motility assay, HUVECs movement along the bottom of a tissue-culture flask was recorded at day 8 after the beginning of treatment with recombinant U94/REP protein (4 μ g/mL). Cells emigrating onto the flask decreased considerably after U94/REP-treatment (F) when compared to untreated cells (E). Data are representative of three independent experiments with similar results.

also with VEGF decreased the number of micro vessels to 83 ± 29 . Lower concentrations of U94/REP (1–2.5 μ g/mL) were still inhibitory, but less effective. U94/REP, at the concentration of 0.5 μ g/mL did not affect rat aorta angiogenesis. This result shows that U94/REP, in addition to interfering with mechanisms underlying spontaneous angiogenesis, also renders aortic rings insensitive to the potent VEGF-induced vasculogenic activity.

Discussion

The findings of our experiments can be summarized in two main conclusions. The first is that LECs are a target for HHV-6

Fig. 6. Effect of U94/REP on microvessel outgrowth in vitro. Rat aortic rings were embedded in type I collagen gels and incubated for 7 days in EBM containing U94/REP (5 μ g/mL) alone (A) or in combination with 20 ng/mL of VEGF (B). Control rings were incubated in EBM in the absence (C) or presence (D) of VEGF. Microvessels structure was observed by phase microscopy on day 7 at magnification, $\times 4$ (A and C) and $\times 2$ (B and D). Numbers in each panel indicate the number of microvessels originating from aortic ring. (Scale bar, 200 nm.)

infection and the second that HHV-6 persistent infection, characterized by expression of the viral gene U94/rep, inhibits angiogenesis of blood and lymphatic ECs. The observation that LECs are a target for HHV-6 in vitro infection extends previous evidence, showing that HUVECs might represent an important target for viral infection. In fact, it was reported that vascular ECs are susceptible to in vitro HHV-6 infection (12), that HHV-6 infects aortic endothelium in vivo (13), and that HHV-6 in vitro acute infection of ECs results in increased secretion of proinflammatory chemokines (14, 15). However, no information is available on the permissiveness of lymphatic endothelium to viral infection, despite the fact that lymphatic tissue, rather than blood compartment, is a more relevant reservoir for virus persistency and replication. The results presented here demonstrate that LECs are susceptible to HHV-6 infection, with low-level productive infection and subsequent establishment of latent infection. In fact, viral antigens were expressed by a small number of infected cells and lytic viral transcripts were present until 14 days p.i., even if they were detectable only by nested PCR (Fig. 1). Production of infectious virus was confirmed by the passage of infection from LECs to lymphoid cells, both by cocultivation, and by testing culture supernatant (Fig. S1). The load of extracellular HHV-6 DNA, decreasing over time, is consistent with the hypothesis that HHV-6 replication in LECs is restricted and occurs over a limited time. Based on the correlation between the considerable amount of copies of cell-associated HHV-6 DNA ($\approx 5 \times 10^7$ in 10^6 cells) and the low number of productively infected cells, as revealed by immunocytochemical staining (≈ 1 –2%), it is possible that HHV-6 infection of LECs is permissive but restricted to specific cell elements. This hypothesis is strengthened by the analysis of U94/rep transcript. In fact, U94/rep is expressed at low levels during productive in vitro infection, but is detected during viral latency (8, 20). It was reported that high levels of U94/REP inhibit HHV-6 in vitro replication (11). Therefore, the simultaneous presence of small amounts of lytic transcripts and high levels of U94/rep mRNA indicate that the intracellular environment of LECs is only partially permissive to viral replication. After few days of infection, lytic transcripts disappear but U94/rep transcript is constantly detected, suggesting that HHV-6 persists in a latent form. Our experiments provide evidence that LECs might serve as a low-level potential HHV-6 reservoir, allowing persistence of virus and release of infectious virions in the microenvironment. HHV-6 is frequently isolated from the saliva of healthy individuals and the viral genome is easily detected in submandibular, parotid and lip salivary glands (21). The abundance of these glands in lymphatic endothelium could suggest the hypothesis HHV-6 detected in saliva might originate from persistently infected LECs.

The most relevant finding of our experiments is that HHV-6 induces loss of angiogenic properties both in LECs and in HUVECs, as shown by the inability to form capillary-like structures and to seal wound scratches (Figs. 5 and 6). Since U94/rep is the most abundant transcript expressed in both cell types, we assayed its effect upon angiogenesis, and showed that the transfection of U94/rep gene fully reproduced the anti-angiogenic effect observed during infection (Fig. 3). The specificity of this phenomenon is supported by the observation that transfection of plasmids deleted in the N- and C- terminal portions of U94/rep failed to inhibit angiogenesis (Fig. S3). The inhibition was even more evident when recombinant U94/REP protein was exogenously added to HUVEC and LEC cultures, and tests of cell migration and capillary-like tube formations were carried out. No toxic or proapoptotic effect was observed, and the angiogenic inhibition was most evident at the concentration of 4 μ g/mL.

Both cell migration and capillary-like formation are in vitro assays that involve endothelial cells present at subconfluent

levels and are indicative of two different angiogenic functions (migration and morphogenesis). However, in vivo angiogenesis involves also sprouting from ECs in preexisting vessels and takes place in an environment composed of multiple cell types. Therefore, we analyzed the effect of recombinant U94/REP in rat aortic ring assay, an ex vivo system that has already been used with success to assess anti-angiogenic agents (22). U94/REP maintained the anti-angiogenic properties also in this assay. These observations are particularly relevant considering that recent reports describe the presence of HHV-6 DNA in myocardial diseases and cardiac dysfunctions (23, 24). A hypothesis that remains to be tested is that also in vivo HHV-6 might be latent in ECs and express U94/REP, significantly impairing physiological angiogenesis, either contributing to the disease or preventing recovery. The mechanism of inhibition remains to be elucidated. The nuclear localization of the protein (Fig. 4) suggests that U94/REP might act indirectly, inducing the expression of specific cell factors. This hypothesis is supported by the observation that U94/REP can regulate gene expression (25), and is confirmed by the observations that it binds both to single-stranded DNA (26) and to human transcription factors (27) and that can prevent oncogenic transformation (25). However, the putative cell factors upregulated or downregulated by U94/REP still remain to be identified. Ifon et al. (28) performed gene array experiments and reported that transfection of U94/rep downregulates angiopoietin-like-4 (ANGPTL4) gene expression in PC3, a prostate cancer cell line. ANGPTL4 has a well characterized role in lipid metabolism, but little is known about its role in vascular biology. It was recently shown that it enhances extravasation, disrupting the integrity of capillaries and mediating tumor cell passage in lung, but not in bone metastases (29). However, at the moment there is no confirmation that ANGPTL4 plays a role in the inhibition of angiogenesis mediated by U94/REP.

The ability of U94/REP to inhibit both blood and lymphatic ECs in their angiogenic properties opens interesting applications. Angiogenesis is a necessary requirement for tumor growth, as tumors cells located 100 μ m away from blood vessels become hypoxic (30) and a tumor cannot grow beyond 1–2 mm without an adequate blood supply (31). The progression of a tumor from a dormant to an active state depends on a series of events, including a switch to an angiogenic phenotype (32). Inhibition of angiogenesis is considered a major step forward in cancer treatment. Current antiangiogenic therapies use inhibitors capable of specifically blocking the activity of a single proangiogenic molecule, but tumors can activate alternate pathways to stimulate the angiogenic process: for example, inhibition of VEGF can induce an increase of bFGF pathway in mice (33). The simultaneous block of different steps of the angiogenic process achieved by U94/REP on the physiology of ECs represents an area susceptible to important developments. On the other hand, lymphangiogenesis in tumors has been associated with increased propensity for metastasis, and has been shown to contribute to malignant progression (34). Therefore, targeting of intratumoral and peripheral lymphangiogenesis represents an important route for antimetastatic approaches. Currently, there is no effective drug able to interfere with lymphomagenesis and prevent cancer metastasis. Recent studies provide evidence that blocking specific growth factors acting on lymphatic vessels might constitute a promising therapeutic approach (35).

The ability of U94/REP to block both vascular and lymphatic angiogenesis opens the way to a promising field of research. In particular, the elucidation of the molecular mechanism responsible for angiogenic inhibition, and in vivo studies on the antiangiogenic properties of U94/REP might lead to important approaches in the potential control of the proliferation of blood and lymphatic vessels.

Materials and Methods

EC Isolation and Culture. Human umbilical vein endothelial cells (HUVECs) were isolated, characterized, and cultured as previously described (14). All of the experiments were performed on third to fifth passage HUVECs. Lymphatic endothelial cells (LECs) were isolated from human lymph node specimens obtained from patients undergoing therapeutic surgical procedures. Approval was obtained from the University of Brescia institutional review board. Informed consent was provided according to the Declaration of Helsinki. Samples were processed as already described (17). Briefly, lymph nodes were minced and digested at with collagenase/dispase (Roche Diagnostics), then seeded on collagen-coated flasks (Biocoat Collagen, BD Biosciences) and cultured in Endothelial Growth Medium (EGM) (Lonza), with 25 ng/mL VEGF-C (RELIAtech). Twenty-four hours later, adherent cells were cultured until 80–90% confluence. ECs were isolated by magnetic separation using anti-human CD31 beads (Miltenyi Biotec). Total CD31⁺ ECs were further cultured, and LECs were purified from ECs by a second step of magnetic separation. Mixed ECs were incubated for 20 min at 4 °C with a mouse anti-human podoplanin mAb (RELIAtech). After incubation, podoplanin⁺ ECs were recovered by magnetic separation with anti-mouse antibodies-coated magnetic microbeads (Miltenyi Biotec). Purified LECs were cultured in collagen coated flasks. All of the experiments were performed between the second and fifth in vitro passage.

Virus Infection. HHV-6A (strain U1102) and HHV-6B (strain CV) viral stocks were generated as previously described (11). Aliquots from the same stock were used for all experiments. HHV-6 viral stock was quantitated by real-time PCR, and the inoculum contained about 2×10^9 genome equivalents per mL. Aliquots of virus stock were inactivated by UV irradiation as described (14). LECs, seeded at 2×10^5 cells and at a confluence of 70%, were infected with 25 μ L of HHV-6 stock. After 3 h of incubation and extensive washes, cells were incubated in complete medium. As negative controls, infections were carried out with the UV-inactivated virus inoculum. The expression of HHV-6 specific antigens was analyzed by immunostaining with monoclonal antibodies reacting to gp60/110 (Chemicon International).

Transmission of Infection from LECs to Lymphoid Cells. Two assays were performed to analyze the production of infectious virus in HHV-6 infected LECs. In the first assay, LECs were infected with cell-free HHV-6A or 6B for 3 h, the inoculum was removed and cells were washed with PBS. After 5 days of incubation, 10^6 infected LECs were cocultured with 10^6 target lymphoid CD4⁺ T cells (JJhan for HHV-6A and SupT1 for HHV-6B). After 5 days of cocultivation, lymphoid cells were collected, cultured in RPMI, and analyzed for HHV-6 infection. The second assay was performed by transferring supernatant from infected LECs to target JJhan or SupT1 cells. Briefly, 10 mL of culture medium were collected from infected LECs, cell debris was eliminated by centrifugation, and supernatant was concentrated on Amicon filters (50 KDa cut off) to 100 μ L final volume. This inoculum was used to infect 10^6 lymphoid target cells.

In both assays, lymphoid target cells were analyzed by real-time PCR and IFA specific for HHV-6 gp116, as described in ref. 11.

PCR and RT-PCR Analyses. DNA was extracted from cell-free culture supernatants or from pelleted LECs by NucliSens miniMAG (BioMerieux). Total RNA was extracted and purified by RNeasy (Qiagen). DNA contamination in RNA preparations was eliminated by three digestion cycles with DNase. RNA was further purified by phenol-chloroform extraction. Transcription of HHV-6 U94, U42, and U22 in infected LECs was analyzed by RT-PCR. RNA was reverse transcribed with random primers, and MULV reverse transcriptase (Applied Biosystems). The resulting cDNA was amplified employing PCR primer sequence and conditions described in ref. 36. Real-time PCR for HHV-6 DNA was performed as described in ref. 37, with the following set of primers/probe: HHV6 U94(+) (5'-GAG CGC CCG ATA TTA AAT GGA T-3'); HHV6 U94(-) (5'-GCT TGA GCG TAC CAC TTT GCA-3'); HHV6 U94 PROBE (5'-FAM-CTG GAA TAA AAC TGC CGT CCC CAC C-TAMRA-3'). The standard curve was generated by amplification of a plasmid containing the targeted HHV-6 sequences. The method had a 6-log dynamic range and a sensitivity of 20 copies/mL.

Transfection. HUVECs (8×10^5 cells) were transfected with 2 μ g endotoxin-free plasmids by nucleofection (Lonza), upon standard conditions. Efficiency of transfection, determined in parallel samples by transfection with pmax-GFP plasmid was about 60% in all experiments.

Cord Formation on Culture Basement Membrane Extract (BME). Two hundred microliters of BME (10 mg/mL) (Cultrex; Trevigen Inc.) at 4 °C were transferred to prechilled 24-well culture plates. Plates were incubated for 1 h at 37 °C,

nucleofected cells were seeded in EGM and cord formation was observed starting from 4 h after cell seeding.

Cloning, Production, and Purification of U94/REP. The full-length U94/*rep* gene was cloned in the pQE-*rep* plasmid, as described in ref. 11, and transfected in the M15 strain of *Escherichia coli*. Selected bacterial clones were induced with 2 mM IPTG, and U94/REP protein was recovered from the insoluble fraction. The protein was solubilized in the presence of 6M urea, and purified by chromatography under denaturing conditions on a hydroxyapatite column. The purity of the protein was >95%. Mock extract were obtained applying the same protocol to bacteria containing only pQE vector sequences. The cloning of U94/*rep* deletion mutants pSR2pH-ΔN (containing the carboxy terminal portion, amino acid 221–490) and pSR2pH-ΔC (containing the amino terminal portion, amino acid 1–320) was described in ref. 11.

Immunofluorescence Assay for U94. Uptake and cellular localization of recombinant U94/REP protein in ECs was analyzed by immunofluorescence, using a rabbit polyclonal serum (pAb HAA/08) raised against a synthetic U94/REP peptide. The peptide NH₂-LSIAKKRIRATRAKDIFIAHC-COOH (U1) corresponding to the hydrophilic domain of the protein, located between the amino acids 271 and 291, its KLH-coupled counterpart and rabbit anti-U1 polyclonal sera (pAb HAA/08) were provided by Primm S.r.l.. Reactivity of sera was tested against U94/REP by ELISA as described in ref. 38. Cells treated with U94/REP, or control cells, were fixed, rehydrated in PBS and incubated for 90 min with the HAA/08 pAb. Slides were washed three times in PBS, and incubated with a secondary anti-rabbit antibody diluted 1:200 in PBS (AlexaFluor, Invitrogen) for 60 min. After three PBS washes, slides were mounted, and samples were observed under a UV light microscope (Nikon Eclipse E600) equipped with a digital camera (DMX 1200).

Wound Sealing Assay. Nucleofected cells were plated into 12-well plates (2×10^5 cells/well) in EGM with 1% FCS. Confluent monolayers were scratched using a 200- μ L pipette tip and serum was increased to 10%. ECs migration was recorded using a DM-IRB microscope system (Leica) equipped with a CCD camera (Hitachi Inc.) connected to a computer via a frame grabber (Matrox Meteor). Analysis of the images were performed using the QWin-lite software (Leica).

Cell Motility Assay. Tissue-culture flasks were coated with collagen I (Sigma). HUVECs were added to the coated flasks at a concentration of 10^5 cells/flask and allowed to adhere by overnight incubation. Flasks were subsequently positioned at an $\approx 20^\circ$ angle so that both the cell-coated surface and the empty surface were immersed in culture medium. Cell motility rates were analyzed at day 8 by measuring the distance from the edge of the flask to the leading edge of the cells.

Aortic Ring Assay. The rat aorta ring assay was performed as described in ref. 19. Briefly, the dorsal aorta was excised from 6-week-old Sprague-Dawley rats. Rings, ≈ 1 -mm thick, were embedded in collagen gel, prepared as described in ref. 19, and then incubated with EBM (Lonza) containing U94/REP (4 $\mu\text{g}/\text{mL}$) alone or in combination with 20 ng/mL of VEGF-A. The plates were maintained in incubation for 10 days. Angiogenesis was quantified by counting the number of microvessels originating from aorta rings (19).

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