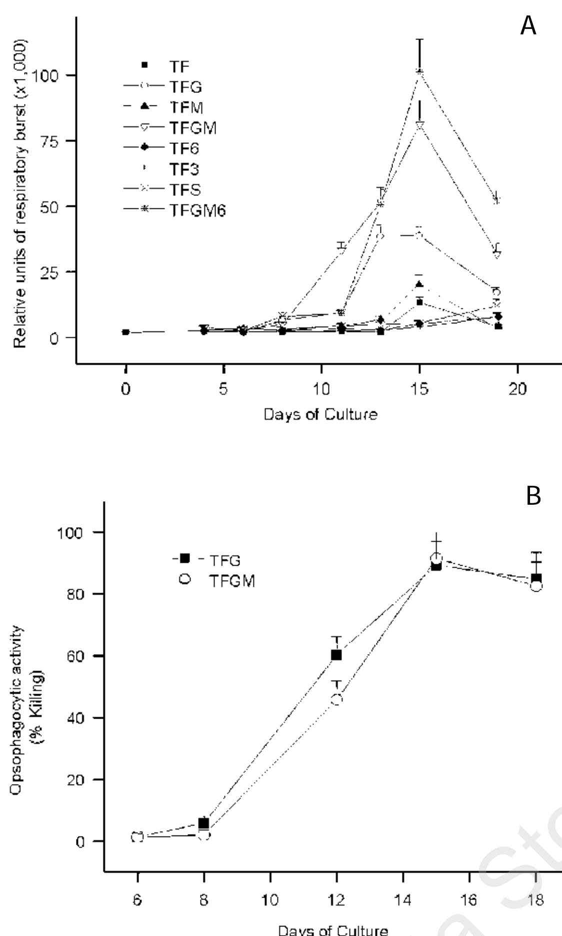

Figure 2. Respiratory burst (A) and microbicidal activities (B) of the cultured cells during *in vitro* differentiation from human cord blood CD34+ cells. Respiratory burst was measured by the chemiluminesce assay. The cultured cells, incubated with 1 μM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) were stimulated with 2 μg/mL of phorbol myristate acetate (Sigma) and the amounts of produced oxygen metabolites were measured by a luminometer (Multi-biolumat LB9505C; Berthhold, Germany). Microbicidal activity was measured by the opsonophagocytic assay for *Candida albicans*. Yeast cells of *C. albicans* were opsonized by incubation with human gamma globulin (Green Cross Co., Seoul, Korea). The opsonized yeast cells were incubated with the cultured cells in a shaking incubator at 37°C for 1 hr. The mixtures were washed and diluted in DW to lyse the cells to release the intracellular yeast, and the numbers of colony-forming units (CFUs) were determined by spread-plating on Brain Heart Infusion agar plates. Microbicidal activity was expressed as % killing, which is defined as (CFUs of control – CFUs experiment)/CFUs of control × 100, where the control is the yeast cells not incubated with the cultured cells. In this experiment, the opsonophagocytic activities were measured at the cell/yeast ratio of 500/1. Data represent mean + SD of four to six separate experiments. T, thrombopoietin; F, flt3-ligand; G, granulocyte colony-stimulating factor; M, granulocyte-macrophage colony-stimulating factor; S, stem cell factor; 3, interleukin-3; 6, interleukin-6.

sion. However, acquisition of each effector function was different depending on the type of cytokine used for *ex vivo* expansion.

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Human herpesvirus-7 infection impairs the survival/differentiation of megakaryocytic cells

In vitro infection of CD61+ megakaryocytic cells with human herpesvirus-7 (HHV-7) induced a drastic increase of apoptosis. Moreover, cells surviving HHV-7 cytotoxicity showed enhanced megakaryocytic maturation with respect to control cultures. These data suggest that HHV-7 reactivation in the bone marrow of HIV-1 infected individuals may contribute to impair megakaryocytopoiesis.

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The human herpesvirus-7 (HHV-7), which shows many similarities with HHV-6 and human cytomegalovirus (HCMV),[1] is a prevalent virus toward which >90% of the population is seropositive by adulthood.[2] The first clinical manifestation clearly associated with primary HHV-7 infection was exanthema subitum,

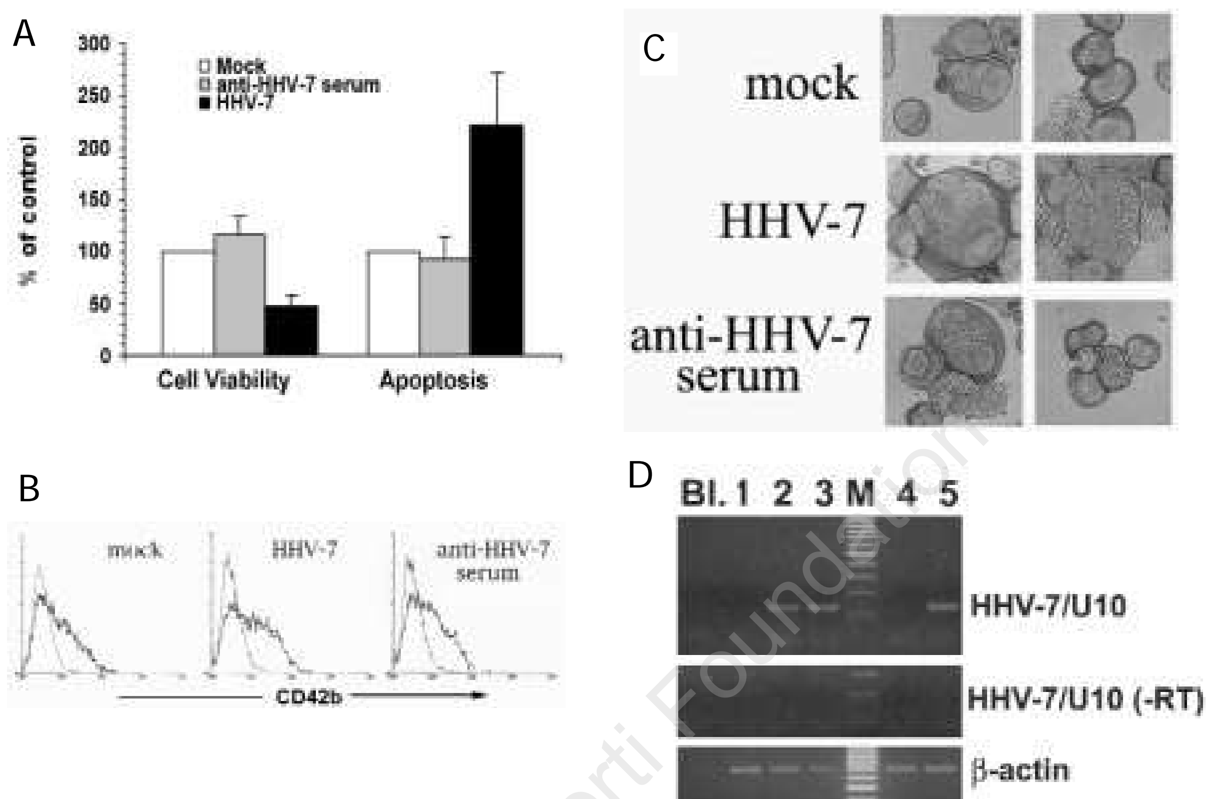

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Figure 1. Effect of HHV-7 on CD61+ megakaryocytic cells. In A, the percentage of viable cells and of apoptotic cells was evaluated by Trypan blue dye exclusion and PI staining followed by flow cytometry analysis, respectively, 8 days post-infection carried out either in the absence or in the presence of anti-HHV-7 serum. Data are expressed as percentage of control (mock-treated cultures) and represent the means ± SD of four separate experiments performed in triplicate. In B, cultures were examined 8 days after HHV-7-infection, carried out either in the absence or in the presence of anti-HHV-7 serum. Representative experiments are shown. Horizontal axis (logarithmic scale), relative surface CD42b expression detected by FITC fluorescence intensity; vertical axis, relative cell number. Negative controls constituted by cells stained with irrelevant isotype-matched monoclonal antibody are shown in each panel (thin lane). In C, May-Grünwald-Giemsa staining was performed on cytospin slides. Original magnification, 40×. D, HHV-7 RNA detection in infected CD61+-megakaryocytic cells. Upper panel: HHV-7 RT-PCR was performed on RNA extracted from either uninfected CD61+ megakaryoblasts (mock; lane 1) or HHV-7-infected CD61+ megakaryoblasts after 3 (lane 2) and 8 (lane 3) days of infection in liquid culture. Lanes 4 and 5: control RNA extracted from uninfected and HHV-7-infected CD4+ SupT1 cells, respectively. Bl: is the blank reaction (samples without RNA); M: molecular weight marker (100 bp, Gibco BRL). Middle panel: as a control for DNA contamination, equal amounts of RNA were used for PCR without template retro-transcription. Lower panel: β-actin amplification was used to confirm comparability of the samples. The ethidium bromide-stained agarose gels of PCR products are shown. Data are representative of three independent experiments from separate infections.

which is sometimes associated with thrombocytopenia.[3-4]

In this study, we investigated the effects of HHV-7 infection on clonogenic megakaryocyte progenitors in semisolid cultures and on cells differentiating along the megakaryocytic lineage in liquid culture. The experiments were carried out on cord blood (CB) CD34+ hematopoietic cells after obtaining maternal informed consent according to the Helsinki declaration of 1975. CB CD34+ cells were isolated using a magnetic cell sorting program (Mini-MACS) and the CD34 isolation kit (both from Miltenyi Biotec, Auburn, CA, USA). Consistently with the previously reported lack of intrauterine transmission of HHV-7,[2] analysis of HHV-7 DNA by polymerase chain reaction (PCR) confirmed that CB CD34+ cells were negative for endogenous HHV-7 infection (*data not shown*).

CD34+ cells were inoculated with cell-free HHV-7, prepared as described elsewhere,[5] and seeded in plasmaclot semisolid cultures, supplemented with stem cell factor (SCF, 50 ng/mL) + thrombopoietin (TPO, 100 ng/mL). The number of megakaryocytic colonies (CFU-meg), scored 14 days following infection, was 34±7 in HHV-7-infected cultures and 27±5 in mock-treated controls (means±standard deviations of four separate experiments), indicating that CFU-meg were not adversely affected by HHV-7,

at least in semisolid assays.

The effect of HHV-7 was next investigated on CD61+ megakaryoblasts, derived from CD34+ cells after 10 days of serum-free liquid cultures,[5] supplemented with SCF+TPO. This approach promotes virus-cell interactions and may more closely resemble *in vivo* infection, as it allows potential secondary infection. Analysis of cell viability performed between 5 and 8 days post-infection revealed that HHV-7 infection induced marked (p<0.01) cytotoxicity on cultured CD61+ megakaryoblasts, mainly due to a significant (p<0.01) increase of apoptosis (Figure 1A). Cells surviving HHV-7 infection showed a brighter expression of the CD42b late megakaryocytic marker than did the mock-treated cultures (Figure 1B), coupled with an increased frequency of mature polyploid megakaryocytes at morphologic analysis (Figure 1C). In fact, there was a significantly higher (p<0.01) number of cells with a diameter greater than 20 μm in HHV-7 cultures than there were in control cultures (53±9% versus 35±8%, respectively, means+SD of four experiments). All these effects were completely abrogated by the neutralizing anti-HHV-7 serum (1:100 dilution, Advanced Biotechnologies, Columbia, MD, USA).

In parallel, we investigated whether HHV-7 infects CD61+ megakaryoblasts and persists in their differentiated progeny. Since the presence of viral DNA could be the consequence of residual virions of the initial inoculum, the occurence of HHV-7 entry was analyzed by reverse transcriptase-PCR, performed on total RNA extracted from CD61+ infected megakaryoblasts at different days post-infection. HHV-7 RNA was detectable at all the time points examined (Figure 1D).

In immunocompromised hosts, human herpesviruses show prompt ability to reactivate generating disseminated infections. In fact, HHV-6 and HCMV have both been associated with many opportunistic pathologic manifestations in AIDS patients (pneumonia, encephalitis, abnormalities of the hematologic picture). In spite of a relative scarcity of definitive evidence to establish the pathogenic potential of HHV-7, it has been demonstrated that reactivation of HHV-7 occurs following bone marrow transplantation[6] and that an increased expression of HHV-7 takes place in lymphoid organs of AIDS patients.[7] For the purpose of this study, it is particularly noteworthy that HHV-7 DNA is present in up to 50% of bone marrow samples of healthy adult donors.[5,8] Moreover, we have previously shown[5] that *in vitro* HHV-7 infection of CD34+ hematopoietic progenitors accelerates differentiation along the granulocytic but not the erythroid lineage, without showing cytotoxic effects. On the other hand, we have demonstrated for the first time in this study that HHV-7 severely impairs the survival of CD61+ megakaryocytic cells, and that the megakaryocytes surviving HHV-7 cytotoxicity show a hastened maturation. Of note, also HCMV selectively inhibits CD42+ megakaryocytes without affecting CFU-meg progenitors or cells of the erythroid and granulocytic lineages.[9] Taken together, the data of Crapnell *et al.*[9] and our present data suggest that megakaryocytes are particularly susceptible to the cytotoxicity of the two closely related herpesviruses, HCMV and HHV-7.

Several factors, such as direct HHV-7 virion/cell interactions and release of cytokines by infected cells are likely implicated in inducing megakaryocyte apoptosis and in promoting maturation along both the megakaryocytic and granulocytic[5] lineages. Due to the central role of megakaryoblasts in the regulation of megakaryocytopoiesis, our data may contribute to explain the occurrence of thrombocytopenia, frequently occurring in patients with HIV-1 disease.[10]

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Complete remission induced by high-dose erythropoietin and granulocyte colony-stimulating factor in acute erythroleukemia (AML-M6 with maturation)

Alternative therapeutic approaches with low dose chemotherapy and differentiative-maturative treatment by growth factors are under consideration for elderly patients with acute leukemia. Two patients with AML-M6 with maturation, one refractory to standard chemotherapy and the other ineligible for cytoxic treatment, obtained complete remission from leukemia using high dose recombinant erythropoietin and granulocyte colony-stimulating factor.

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In the last years the use of recombinant human erythropoietin (rHuEpo) has increased greatly. Beneficial effects have been reported in myelodysplasia[1] and in avoiding chemotherapy-